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Competitive Inhibition of Succinoxidase by Acetylene Dicarboxylate.* (25027)

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Among many compounds tested as inhibitors of the succinoxidase system there are a number of dicarboxylic acids closely related to succinate which inhibit competitively, *e.g.*, malonate, oxalacetate, glutarate, etc. One compound that has been largely overlooked except for a brief report by Dietrich *et al.*(1) is the acetylene derivative, acetylene dicarboxylic acid (ADC). Since this compound has been used to prepare tetradeuterosuccinic acid and could conceivably be present in small quantity in the product, it was of interest to reexamine its inhibition of the dehydrogenation of succinate.

Methods. Dipotassium ADC was prepared by neutralizing the commercial monopotassium salt with KOH, and precipitating K_2ADC from a 25% solution by addition of 5 volumes of ethanol. After 3 such precipitations the salt was dried *in vacuo*. Succinoxidase was assayed by the method of Schneider and Potter(2). Homogenized rat kidney was used as the source of the enzyme system. The mechanism of inhibition was studied both by varying the substrate concentration in the presence of constant inhibitor concentration, and *vice versa*. The methods of Lineweaver and Burk(3) and Goldstein(4) were used to analyze the nature of the inhibition.†

Results. The order of addition of substrate and inhibitor is of considerable importance (Fig. 1). When ADC was added 20 minutes after substrate and enzyme were mixed, rate of oxygen consumption decreased slowly; and only after 5 hours did it fall to the level observed when the substrate was added after the inhibitor and enzyme were mixed.

Fig. 2 is a Lineweaver-Burk plot of the

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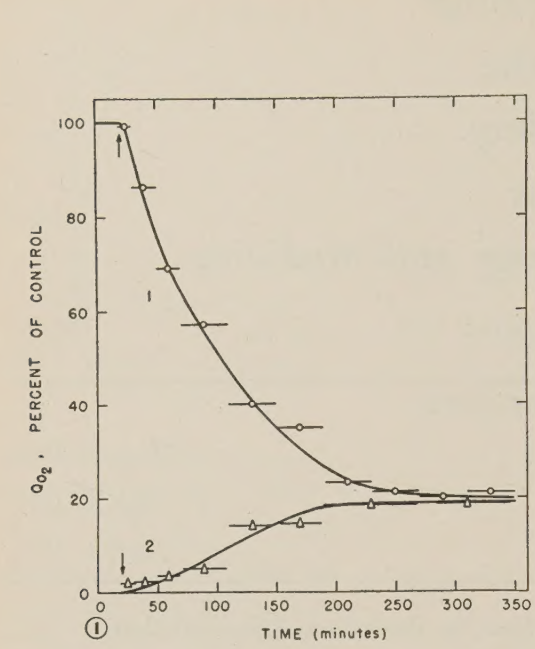
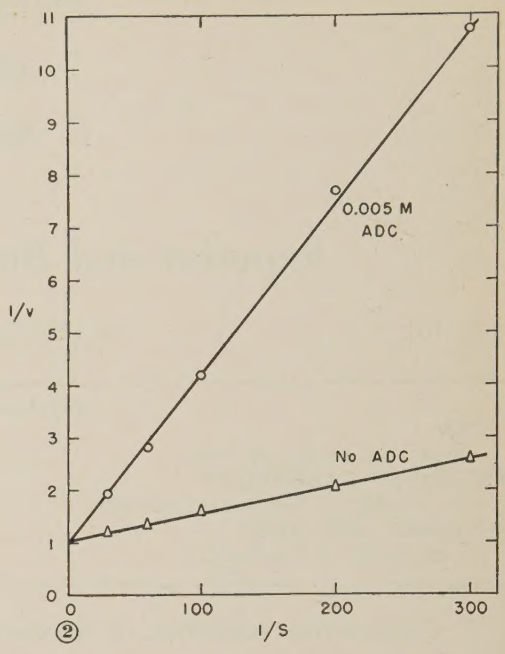
† The following symbols are used: K_S, dissociation constant of enzyme-inhibitor complex (Michaelis constant); K_I, dissociation constant of enzyme-ADC complex; S, molar concentration of succinate; I, concentration of ADC; v, rate of oxygen consumption (extrapolated to zero time from 10, 20, and 30 minutes readings); V_m, theoretical maximum rate of oxygen consumption (at "infinite" substrate concentration).





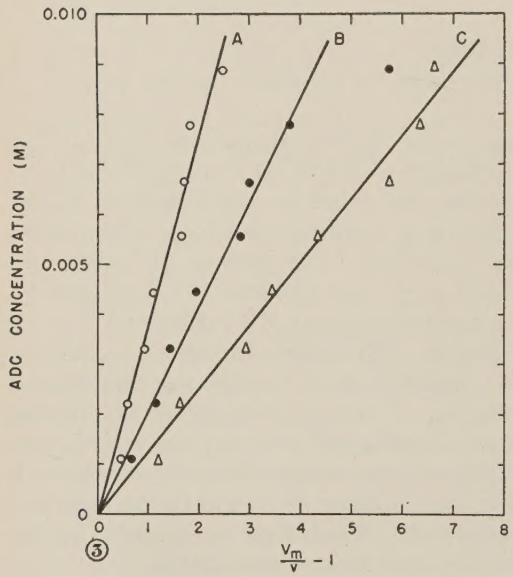


FIG. 1. Effect of order of addition of succinate and ADC. Curve 1, succinate added at zero time, ADC after 20 min. (arrow); Curve 2, ADC added at zero time, succinate after 20 min. Ordinate is rate of oxygen consumption, as % of control, over a time interval the length of which is indicated by horizontal lines through each point. Concentrations of succinate and ADC were 0.083 and 0.0033 M, respectively.

FIG. 2. Inhibition of succinoxidase by ADC (Lineweaver-Burk plot). Both succinate and ADC were added to the enzyme simultaneously. The slope with no ADC $(K_S/V_m) = 0.00465$; the slope with 0.005 M ADC $(K_S/V_m)(1 + I/K_I) = 0.03263$.

FIG. 3. Inhibition of succinoxidase by ADC (Goldstein plot). Succinate, ADC, and enzyme incubated 18 hr at 1°C before assay. Curve A: Succinate conc. 0.0833 M; slope $(S(K_I/K_S)) = 0.00377$. Curve B: Succinate conc. 0.05 M; slope = 0.00209. Curve C: Succinate conc. 0.0167 M; slope = 0.00063. In curve C, the values of $(V_m/v - 1)$ have been divided by 2 to conserve space.

inhibition produced by simultaneous addition of inhibitor and substrate. From these data K_S was calculated to be 0.00412, and K_I 0.00081, about a 1:5 ratio. In this experiment substrate concentration was varied, while inhibitor concentration remained constant. A very similar value, $K_I = 0.00079$, was obtained in an experiment with constant substrate and variable inhibitor concentration; equation $7A_IA_S$ of Goldstein was used in this experiment. There is no doubt that the inhibition is competitive.

Considerably lower values for K_I (1/90 to 1/30 the value for K_S) were obtained when the substrate was added after inhibitor and enzyme were mixed, although a 3-fold variation in K_I values was observed in different experiments. Therefore, the substrate, in-

hibitor, and enzyme were mixed and stored at 1°C for 18 hours before assay. Fig. 3 illustrates the results of such an experiment in which 3 concentrations of succinate and 8 concentrations of ADC were employed. From these data, K_I was estimated to be 0.000171 ± 0.000063, about 1/25 that of K_S.

Discussion. Dietrich *et al.*(1) reported that ADC, like malonate, was a competitive inhibitor of pigeon breast succinoxidase. However, their data leave something to be desired, since the best straight line which can be drawn in their Lineweaver-Burk plot of reaction velocity in the absence of inhibitor gives a *negative* value for $1/V_m$. From another experiment in their paper, a value of $K_S = 0.03$ was estimated; this value is some 20 times higher than that observed by other investigators(see(5)). Using this value of K_S, one can calculate that K_I for both ADC and malonate is about 0.005-0.007, so that the ratio of K_S to K_I is about 5. Other workers have reported values of 30 to 50 for the K_S/K_I ratio for inhibition of succinoxidase by malonate(5,6). Finally, Dietrich *et al.*(1) did not specify the order of addition of succinate and ADC; on the basis of their K_S/K_I ratio, it would seem that the inhibitor was added to the enzyme either at the same time or after the substrate was added, since this ratio of 5 was what we observed in the experiment reported in Fig. 2.

Thus under the conditions presumably employed by Dietrich *et al.* or by us in the experiment shown in Fig. 2, ADC is a competitive inhibitor of succinoxidase with an affinity about 5 times that of succinate. However, when the components of the system are incubated overnight prior to assay, the affinity of ADC appears to be about 25 times that of succinate. ADC is thus about as effective as malonate and considerably less effective than oxalacetate as an inhibitor of succinic dehydrogenase.

On the basis of experiments similar to that reported in Fig. 1, we have estimated using Goldstein's equation 16A(4), the rate constants for dissociation of the enzyme-substrate complex, $ES \rightarrow E + S$, and of the enzyme-inhibitor complex, $EI \rightarrow E + I$. The absolute values are of doubtful value because of the presence of other reactions; however, on a relative basis, the former rate was 44 times the latter. This difference thus explains why a long incubation time is necessary to obtain more nearly correct values for the ratio of dissociation constants, K_S/K_I.

Summary. Acetylene dicarboxylate (ADC) is a competitive inhibitor of succinoxidase. The apparent dissociation constant of the ADC-enzyme complex is about 1/5 that of the succinate-enzyme complex when the inhibitor is added to the enzyme after or along with the substrate. However, when the enzyme, inhibitor, and substrate are incubated 18 hours before assay, the value of K_I is about 1/25 that of K_S.

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Search for Illness Due to Adenovirus Type 4 Among College Dormitory Freshmen.* (25028)

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Illness due to adenovirus type 4 has been described in military(1,2,3) but not in civilian populations(4). As part of continuing interest in epidemiology of this disease, the present study was designed to search for infection with adenovirus type 4 in groups of college freshmen living in dormitories. This was considered a logical population to explore because it resembled in certain aspects the military recruit population in which illness due to adenovirus type 4 had been characteristically described(1). Both included a large number of young adults from a variety of geographical locations, living in close personal contact, newly exposed to one another, and engaged in similar activities.

Methods. Freshmen dormitory students from Case Inst. of Technology (men) and from Adelbert (men) and Mather (women) Colleges of Western Reserve Univ. donated blood specimens during first week in dormitories and again at end of first school year, 1955-56. One hundred and two students voluntarily complied. In addition, acute and convalescent phase serum samples were collected from 24 freshmen with respiratory tract infections at U.S. Military and Naval Academies during academic years 1955-56 and 1956-57. Serum specimens were stored at –20°C, and pairs of sera from each student were subsequently analyzed for changes in titer of complement-fixing antibodies against an antigen prepared from adenovirus type 4. Pairs of sera that revealed increase in titer during interval of study were reexamined for presence of neutralizing antibodies against adenovirus types 1, 2, 3, 4, 5, 6, and 7. The methods used have been described(2,5,6).

Results. Pairs of serum specimens were available from the 102 students, the time span between individual pairs being 7½ months in 99 students and 3 months in 3 students. The 102 students comprised 25% of the freshman dormitory population and 11% of total freshman class in the 2 schools. Pairs of sera samples were available from 24 students from U.S. Naval and Military Academies. Occurrence of increase in titer of complement-fixing antibodies was sought. The results are summarized in Table I. An increase in titer was noted in specimens of 3 students, 2 from Adelbert College, Western Reserve Univ. and 1 from Case. None of the 3 showed increase in titer of serum neutralizing antibodies against adenovirus types 1, 2, 3, 4, 5, 6, and 7. None of the paired serum samples from the Service Academies demonstrated significant rises of complement-fixing antibody. The sera of one individual from Service Academies showed a 1-tube rise in complement-fixing antibody and a 3-tube rise in neutralizing antibody to type 7 adenovirus, but no antibody rise to type 4 adenovirus.

Discussion. On the basis of epidemiologic studies during World War II the Commission on Acute Respiratory Diseases described a febrile respiratory illness among military recruits(1). The illness became known as "acute respiratory disease (ARD)." More recently, the etiology of the disease was ascribed principally to adenovirus type 4, although adenovirus type 7 was shown to be a cause, and adenovirus types 3 and 14 possible causes of such illness(2,3,7,8). With development of methods for detection of serum antibodies, additional tools became available for

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TABLE I. Complement-Fixation Titers against Adenovirus Type 4 in Dormitory Freshmen of Case Inst. of Technology (1955-56), Western Reserve Univ. (1955-56), and U. S. Military and Naval Academies (1955-56, 1956-57).

Institution	No. of dormitory freshmen	No. tested	Complement-fixation titer		
			Increase	No increase	Anticomplementary
Case Institute (men)	264	74	1	69	4
WRU					
Adelbert (men)	63	13	2	11	0
Mather (women)	74	15	0	14	1
Service academies		24	0	24	0
Totals		126	3	118	5

the hitherto unsuccessful search for presence of the disease among civilians(9,10,11).

The antibody analyses in the present study revealed no instance of infection due to adenovirus type 4 in 102 dormitory freshmen during the school year 1955-56, nor in 24 students from U.S. Military and Naval Academies, who experienced respiratory infections during academic years 1955-56 and 1956-57. A significant rise in type 7 adenovirus neutralizing antibody was demonstrated in the paired sera of one student from the Naval Academy: a 1-tube rise in complement-fixing antibody was demonstrated in sera from this individual. It is believed that the sample of students in our study was adequate to indicate the minor role of infections due to adenoviruses in freshman dormitory population. It is of interest that paired sera from an additional 29 students from the Naval Academy, all from upperclassmen, also failed to show a rise in complement-fixing antibody.

Absence of increases in neutralizing antibody titers against adenovirus types 1-7 in sera of the 3 students with increases in complement-fixing antibody titers signified that the agent responsible for the increase in titers was immunologically different from but related to adenovirus types 1-7. This may be explained by previously reported observation that complement-fixing antibody against viruses of adenovirus group is group-specific, while neutralizing antibody is strain-specific (11,12,13,14).

Summary. A search was made for presence of infection due to adenovirus type 4 in dormitory freshmen of Case Inst. of Technology, Western Reserve Univ., and U.S. Military and Naval Academies. Antibody analyses revealed no instance of such infection in 126 students. Three students had rises in serum titers of complement-fixing antibodies against adenovirus type 4; however, none of the 3 had rises in serum titers of neutralizing antibodies against adenovirus types 1, 2, 3, 4, 5, 6, and 7. One student from the Naval Academy who had only a 1-tube rise in complement-fixing antibody developed a significant rise in neutralizing antibody against type 7 adenovirus.

We express sincere appreciation to students and personnel of Case Inst. of Technology, Western Reserve Univ., and U. S. Military and Naval Academies. We wish to thank C. H. Cramer, R. A. Griffin, E. T. Hastings, E. Walker, C. L. Hudson, K. B. McEachron, Jr., R. W. Waite, J. B. Stapleton, J. N. C. Gordon, and H. L. Ley, Jr.

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High Activity of Potent Analgesics on Conditioned Rat Tranquilizer Test. (25029)

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Inhibition of conditioned avoidance-escape response in rats has been widely used as one of the pharmacological tests for tranquilizing agents of reserpine and chlorpromazine types (1,2). Furthermore, it has been reported that blocking of this conditioned response is also produced by morphine(1). To investigate the possible correlation between analgesic potency and inhibition of this conditioned response with compounds related to morphine, we have now compared 3 analgesics and 2 well known tranquilizers on this test. The 2 tranquilizers used were chlorpromazine hydrochloride (Thorazine Hydrochloride ampuls, 25 mg/cc) and perphenazine (Trilafon ampuls, 5 mg/cc). The 3 analgesics were the following: Codeine Phosphate, U.S.P., a moderately potent analgesic; Morphine Sulfate, U.S.P., a highly potent analgesic; and *l*-14-hydroxydihydromorphinone hydrochloride (HDM hydrochloride, Numorphan Hydrochloride), a new and very highly potent analgesic. The latter has been reported 12 to 18 times as active as morphine sulfate by injection in mouse analgesia studies(3,4) and 8 to 10 times as active in clinical studies(3,5,6,7).

Methods. The apparatus and method for conditioned avoidance-escape response were essentially those described by Cook and Weidley(1). Rats were conditioned to jump onto a wooden pole at the sound of a buzzer to avoid a mild electric shock from metal rod floor. If the conditioned response to buzzer was blocked, the electric shock was then turned on to make the rat jump to the pole by unconditioned response. This procedure demonstrated that blocking was specifically of the conditioned response and was not attributable to general depression or motor weakness. Female albino rats of CF strain (Carworth Farms), weighing 120-220 g, were used. In a confirmatory experiment full-grown female albino rats of a different strain (Supplee-Wistar), weighing 200-300 g, were used. Analgesic activity was determined by the mouse hot-plate method(8) in CF-1 male mice (Carworth Farms), weighing 20-28 g. The ED_{50} values were calculated graphically(9).

Results. In the first experiment on conditioned response, the 5 drugs were administered subcutaneously to CF rats, 40 rats being used for each drug. The 3 analgesics all demonstrated blocking activity on this test, although of varying degrees (Table I). The very highly potent analgesic HDM was even slightly more active than perphenazine, one of the most potent tranquilizers in clinical use. HDM was about 1.6 times as potent as perphenazine on this test, calculated as base. Essentially similar results were obtained when the 2 compounds were compared again in older rats of the Supplee-Wistar strain, although the

TABLE I. Inhibition of Conditioned Response, by Subcutaneous Injection.

Drug	$ED_{50} \pm$ S.E.,* mg/kg	Potency
Chlorpromazine	7.0 ± .7	1.0
Perphenazine	.42 ± .05	17
Codeine	49 ±7	.14
Morphine	7.4 ± .8	.95
HDM	.26 ± .01	27

* Calculated as base.

TABLE II. Comparative Potencies on Conditioned Response Blocking and Analgesia Tests, by Subcutaneous Injection.

Drug	Conditioned response blocking		Analgesia		
			Mouse		
	$ED_{50} \pm$ S.E., mg/kg	Potency	$ED_{50} \pm$ S.E., mg/kg	Potency	Man potency
Codeine phosphate	70 ± 9	.14	18 ± 2	.12	.15
Morphine sulfate	9.9 ± 1.0	1.0	2.2 ± .4	1.0	1.0
HDM hydrochloride	.31 ± .01	32	.12 ± .02	18	10

latter were more sensitive and showed blockage of conditioned response at lower dosage baseline. In this confirmatory experiment values for blocking $ED_{50} \pm$ S.E. were perphenazine 0.21 ± 0.02 and HDM 0.16 ± 0.02 mg/kg, HDM again appearing slightly more potent than perphenazine.

The patterns of action differed somewhat in that the analgesics showed a more rapid onset of blocking than did the tranquilizers. Average times of onset at approximately the ED_{50} were as follows: chlorpromazine 114, perphenazine 141, codeine 36, morphine 32, and HDM 39 minutes. However, average duration of action of the tranquilizers was longer than that of the analgesics, as follows: chlorpromazine 276, perphenazine 246, codeine 152, morphine 140, and HDM 141 minutes.

Analgesic activities of codeine phosphate, morphine sulfate, and HDM hydrochloride were determined in mice, using 80 mice for each drug. In Table II the 3 analgesics are compared for potency by subcutaneous administration in the conditioned response blocking test, in mouse analgesia, and with reported figures for analgesia in man(3,6,10). As the clinical studies cited were always carried out in comparison with morphine sulfate dosage, potency values in the Table have been calculated as weights of salts and compared with morphine sulfate as 1. There is a rough correlation between rat conditioned response blocking potency and both mouse and human analgesic potencies. Thus, on the 3 different tests codeine phosphate had only 0.12-0.15 of the potency of morphine sulfate, whereas HDM hydrochloride was 10-32 times as potent as morphine sulfate. Parenthetically, chlorpromazine and perphenazine are not considered to produce analgesia, although they do potentiate analgesics, *e.g.,* morphine. Unlike the 3 analgesics, in the mouse hot-plate test chlorpromazine and perphenazine showed an analgesic response only at dosages which also produced obvious sedation and central nervous system depression.

When HDM was administered orally, its potency on mouse analgesia test was greatly reduced, being only 0.07 of its subcutaneous potency. Similarly, its oral potency on conditioned response blocking test was also markedly reduced to about 0.02 of its subcutaneous potency, whereas oral potency of the tranquilizer, perphenazine, was reduced much less, *i.e.,* only to about 0.2 of its subcutaneous potency. Thus, although HDM was about 1.6 times as active as perphenazine on the conditioned rat test subcutaneously, it was only 0.1 as active orally.

Activity in the conditioned response blocking test was also shown by synthetic narcotic analgesics, such as meperidine. However, sodium salicylate appeared inactive both subcutaneously and orally.

Although the significance of high "tranquilization" test activity of potent analgesics is conjectural, it is interesting that a rough correlation between conditioned response blocking activity and analgesic activity was found in these 3 analgesics of widely differing potencies. This tranquilizer-like action may be related to the hypothesis advanced(11) that reduction of pain-anticipatory anxiety is a necessary action that a drug must exert to be a potent analgesic.

Summary. The analgesics codeine, morphine, and *l*-14-hydroxydihydromorphinone (Numorphan) produced inhibition of the conditioned avoidance-escape response in rats, a widely used tranquilizer test. The potencies in blocking conditioned response correlated

roughly with analgesic potencies. The very potent analgesic, *l*-14-hydroxydihydromorphinone, exhibited by subcutaneous injection slightly more conditioned response blocking activity than perphenazine, one of the most active tranquilizers now in clinical use.

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Induction of Immunological Tolerance to Male Skin Isografts in Female Mice Subsequent to Neonatal Period.* (25030)

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Histo-incompatibility to male skin isografts in females of some inbred strains of mice, particularly those of A and C_{57}Bl strains, was first reported by Eichwald and Silmser(1) and later confirmed by others(2-8). Some investigators believe that this phenomenon occurs in most strains(6,9). Eichwald *et al.*(10), Hauschka(11), and Snell(12) have interpreted rejection of male skin by female of same strain as an immune response due to recipient's lack of donor's isoantigen which originates from a gene on the Y chromosome. The immunological nature of this phenomenon has been further supported by recent findings of Billingham *et al.*(4) and Mariani *et al.*(5) which demonstrate that induction of acquired tolerance to overcome the histo-incompatibility to male skin isografts, can be produced in females of specific strains by injecting them at birth with viable spleen cells obtained from isologous adult male donors. Our findings indicated that the age of both recipient and donor at time of skin isotransplantation influences the frequency of male skin rejection by females of same inbred strain (19). In essence, these investigations demonstrated that weanling females would more frequently accept male skin than would older females of the same strain. As a result 2 studies were undertaken: (1) To determine the possibility of inducing a state of tolerance in female mice of A and C_{57}Bl (subline 1) strains by I. V. injection of viable male spleen cells during post neonatal period and (2) to determine whether tolerance to male skin could also be induced in A and C_{57}Bl (subline 1) females by joining them in parabiosis with isologous males.

Method. In a first series of experiments 2 groups of A and C_{57}Bl (subline 1) strains of mice pre-treated with spleen cells were used. Spleen cells were prepared according to method previously described(18,20). Approximately 20 million viable male spleen cells suspended in 0.2 cc of Ringer-Locke's saline solution were injected intravenously into the tail vein of A females 30-47 days old and 92 days old recipients. Cell viability was determined by the eosin method(21). Similar numbers of spleen cells/mouse were also transferred from isologous adult C_{57}Bl (subline 1) males to C_{57}Bl (subline 1) females of 32-48

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TABLE I. Induction of Acquired Tolerance in Female *A and $C_{57}Bl$ (Subline 1)* Strains following Intravenous Injection of Viable Male Spleen Cells.

Strain	Age when inj. ♂ spleen cells	Age when grafted ♂ skin (days)	Successful grafts No. takes/Total No. grafted	%
A	0–24 hr*	20–34	23/24	96
$C_{57}Bl$ (subline 1)	"	30–50	18/20	90
A	30–47 days†	60–72	20/23	87
A	92	110	12/13	92
$C_{57}Bl$ (subline 1)	32–48	60–61	9/ 9	100
$C_{57}Bl$ (")	69–91	110	8/10	80
A	Non-inj.	29–62	23/40	58
$C_{57}Bl$ (subline 1)	"	29–70	3/35	9

* Approximately 2-4 million viable male spleen cells inj./mouse.
† Approximately 20 million viable male spleen cells inj./mouse.

days old and 69-91 days old. Approximately 30 days following intravenous injection of spleen cells, the 30-47 days old A females and the 32-48 days old $C_{57}Bl$ (subline 1) females were subjected to male skin isograft. The 92 days old A females and 69-91 days old $C_{57}Bl$ (subline 1) females were grafted when these animals reached 110 days. A 2 x 2 cm full-thickness abdominal skin graft from a male donor was dissected free of subcutaneous fat and transferred to dorsal surface of female recipient as described by Martinez *et al.*(18). The graft was turned 180° to facilitate determination of subsequent success or failure of the graft, since hair on a successful graft grows in the opposite direction to hair growth of host. Interrupted 5-0 black silk sutures were used to bring the skin transplant in close union with surrounding host tissues. In the second experiment coelomic parabiosis was performed between members of both A and $C_{57}Bl$ (subline 1) mice as follows: male with female and female with female. For mice of A strain the 58-93 days old females were placed in parabiosis with 55-127 days old males. Skin grafts were exchanged between respective members of each parabiotic pair from 7 to 14 days following establishment of parabiosis. The $C_{57}Bl$ (subline 1) 53-101 days-old females in parabiosis with 51-72 days old males were also subjected to similar exchanges of skin transplants 9 to 12 days subsequent to union. Control groups for both strains consisted of females placed in parabiosis with females which later received skin transplants from male donors of appropriate ages. The parabiosis method was a modification of that described by Sauerbruch and Heyde(22) and Bunster and Meyer(23) briefly as follows: After hair removal from opposite lateral sides of each pair, an incision was made including skin and muscles from base of ear to proximal insertion of femur. To achieve confluence of coelomic cavities, the incision was extended into the peritoneal cavity from the lower edge of last rib to the iliac crest. The peritoneums of the 2 animals were conjoined and closed with 5-0 plain catgut continuous sutures, while skin and muscles of both dorso-lateral and ventro-lateral flaps were closed with Michel skin clips left in place for 7 days. No dressing or bandage was used. Skin grafts were performed on parabiont mice using the same procedures as before. All grafted animals were placed in individual plastic cages and observed for 4 to 12 months.

Results. The results of grafting male skin to female mice which had been given I. V. injections of viable male spleen cells in the weanling stage are summarized in Table I. In A strain females injected at 30-47 days of age, 87% of isologous male skin grafts were successful. Similarly in A females injected with male spleen cells at 92 days of age, successful transplantation of male skin occurred in 92%. Comparable results were obtained in $C_{57}Bl$ (subline 1) strain females since male isograft acceptance for those injected at 32-48 days and those treated at 69-91 days of age was 100% and 80% respectively. The differences in incidence of male skin isograft acceptance between pre-treated females of both strains and corresponding non-treated controls are statistically significant. Data on male skin isograft acceptance in females injected at birth with viable male spleen cells are in-

TABLE II. Induction of Acquired Tolerance in Female *A and $C_{57}Bl$ (Subline 1)* Strains to Male Skin Isograft following Parabiosis.

Parabiosis groups	Age when joined in parabiosis (days) ♀	♂	Time in parabiosis (days)	No. female parabionts accept. male skin graft*	%
A ♂ ↔ A ♀	58– 93	55–127	7–14	19/21	90
$C_{57}Bl$ ♂ ↔ $C_{57}Bl$ ♀	53–101	51– 72	9–12	13/15	87
A ♀ ↔ A ♀	60		14–15	0/ 5	0
$C_{57}Bl$ ♀ ↔ $C_{57}Bl$ ♀	62–104		9–15	0/ 9	0

* $\frac{\text{No. takes}}{\text{Total No. grafted}}$.

cluded in Table I.

Table II shows results obtained in parabiont animals. The female member of the pair, placed in parabiosis with isologous males in both A and $C_{57}Bl$ (subline 1) groups of mice, accepted skin isografts from the male partner much more frequently than did control females which had been placed in parabiosis with another female of same strain. In fact while incidence of acceptance of male skin isograft was 90% in the A strain and 87% for the $C_{57}Bl$ (subline 1) strain, none of the control groups of either strain accepted the male skin isotransplant. Fig. 1 shows a successful male skin isograft on a female of A strain in parabiosis with an isologous A male.

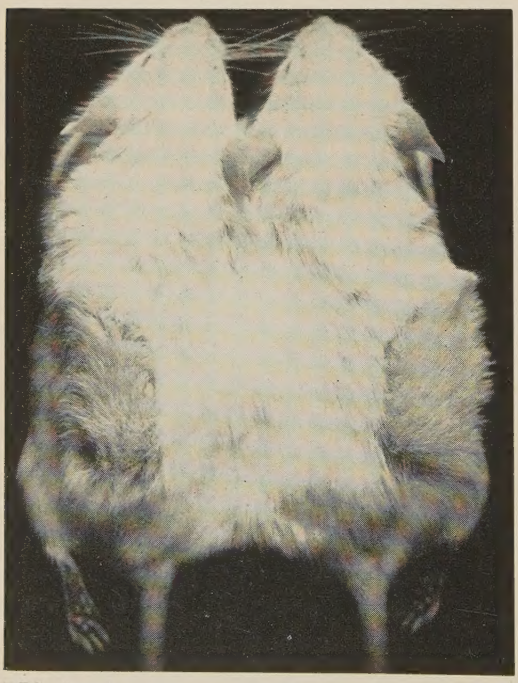

FIG. 1. A ♂ ↔ A ♀ parabiosis. Left animal is A ♀ with A ♂ skin graft. Right animal is A ♂ with A ♀ skin graft.

Discussion. The results of these studies indicate that the immunological incompatibility existing between male and female mice of both A and $C_{57}Bl$ (subline 1) strains can be overcome not only in the neonate but also in older mice by injecting females intravenously with adult viable male spleen cells. Furthermore, parabiotic union between male and female mice during adult life will also apparently overcome resistance of female partner to transplantation of male skin in both strains studied.

Approximately the same incidence of successful male skin isotransplantation was achieved when female recipients of A strain were treated with male spleen cells at age 30-47 days or 92 days as when recipients were treated at birth (87%, 92% and 96% respectively). Similar results were obtained in female mice of the $C_{57}Bl$ (subline 1) strain (Table I). This would indicate that the limitation imposed by age on induction of immunological tolerance in females to male skin isografts might be only of relative importance. In addition, the fact that induction of this phenomenon in mature female recipients was achieved in both A and $C_{57}Bl$ (subline 1) strains of mice indicates that this is not limited to only one strain of mice.

Induction of tolerance by joining females in parabiosis with adult isologous males of approximately the same age is of interest from two standpoints. First, it supports the concept that older mice can be made tolerant if appropriate circumstances can be achieved, as indicated in the first experiments. Second, it indicates that antigens, presumably contained in cells and capable of inducing a state of tolerance, are circulating in sufficient number and are being transferred between both

members of the parabiotic pair.

The results reported in which female mice are made tolerant of male skin isografts when treated with male spleen cells at an age well beyond the neonatal period can be related to relative weakness of histocompatibility barrier between males and females of these strains. That female mice will accept an intravenous injection of male spleen cells and develop a high degree of tolerance at an age when they are capable of rejecting a male skin isotransplant seems clear but is not explained. This could be a function of a greater capacity of spleen cells to overcome an immunological barrier or even a relative lack of availability of transplantation antigens in spleen cells administered by this route. This deserves further study.

Experiments designed to elucidate length of time that parabiosis must exist prior to development of tolerance and duration of tolerance following separation of parabiotic union are of importance and are being studied. Studies of capacity of female mice specifically immunized against male skin isografts to develop immunological tolerance produced by either of these methods would be also of interest.

Although our observations are somewhat difficult to interpret, they do not permit retention of the view that induction of immunological tolerance is a phenomenon limited to the neonatal period (13-18). They also seem to provoke further speculation concerning possible relationship between immunological tolerance and immunological paralysis (24), specific immunological unresponsiveness in adults (25), specific immunological unresponsiveness facilitated in adult animals by irradiation (26, 27) or other inhibitors of the immune response. (28).

Summary. (1) Acquired tolerance of male skin isografts has been produced in females of inbred A and C_{57}Bl (subline 1) strains, over a wide age range, either by injection of viable male spleen cells or by placing females in parabiosis with males. (2) Attention is directed to the importance of age and degree of histocompatibility difference in susceptibility to induction of acquired tolerance. (3) The implications of these observations are discussed.

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Adrenocorticotropic Activity of Alpha Melanocyte Stimulating Hormone (α-MSH).* (25031)

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During the past 2 years α-MSH has been isolated and its structure determined(1,2). It is a tridecapeptide with an amino acid sequence identical to that of Corticotropin-A except that the N-terminal amino acid (serine) is acetylated and the C-terminal valine is in the amide form. A preliminary report of Lerner(3) stated that α-MSH did not possess corticotropic activity as measured by adrenal ascorbic acid method. Recently, Steelman *et al.*(4) have reported a simplified method for isolation of α- and β-MSH using carboxymethyl cellulose. Since ACTH-peptides have high adipokinetic activity(13, Steelman and Guillemin, to be published), α-MSH was assayed using the Payne(5) method. Doses as high as 1000 μg did not significantly increase liver fat in the mouse, whereas 1-3 μg of highly purified ACTH were active.

Materials and methods. ACTH activity of α-MSH(4) has been determined by the *in vitro* method of Saffran and Schally(6) and *in vivo* in 24 hr-hypophysectomized rat using levels of plasma free corticosteroids(7) and depletion of adrenal ascorbic acid(8) as criteria of adrenocorticotropic activity. In both types of assays USP Reference Standard Corticotropin was used as Standard. The *in vitro* assay is a 4-point design with duplicate observations for $U_{1,2}$ $S_{1,2}$; the *in vivo* assays were 4-point designs with 4 or 6 replicate observations per dose of each $U_{1,2}$ and $S_{1,2}$. The crude data of all assays were analyzed by variance analysis and factorial analysis with special emphasis placed on a study of degree of parallelism of the U/S log dose-response regression lines(9).

Results. Results are reported in Table I. All preparations of α-MSH tested exhibited corticotrophic activity. It appears that as degree of purification of α-MSH increases, so does corticotrophic activity. The corticotrophic activity was retained after incubation of the material at room temperature for 16 hr in 0.1N KOH or 0.1N HCl. Storage in deep freeze (–20°C) in dry state for 10 weeks did not alter significantly the corticotrophic or the melanophoretic activity.

Discussion. As reported elsewhere(4), the most purified MSH preparations used here exhibit less than 0.5 U/mg vasopressor activity when tested against the USP posterior lobe reference Standard. Assuming that this vasopressor activity is due to contamination with one of the vasopressins, evidence has been offered previously(7) which would preclude that the corticotrophic activity seen here could be attributed to either lysine or arginine vasopressin at the dose which could account for this contamination. The corticotrophic activity seen here seems to be truly inherent to the molecule of α-MSH.

Furthermore, in view of the absolute parallelism of the response curves, corticotrophic activity of α-MSH cannot be distinguished from that of ACTH USP Standard whether one measures Δ^4-3-keto steroids *in vitro*, plasma H_2SO_4-fluorescent steroids or adrenal ascorbic acid depletion *in vivo*. Considering such a parallelism of the otherwise linear log dose *vs.* response functions for unknowns and standard it appears that the corticotrophic activity of α-MSH cannot be related to the type of "non-specific" effects reported by Roberts(10) upon addition of large amounts of plasma proteins to adrenal fragments *in vitro;* the mathematical prerequisites for true corticotrophic activity as obtained here were never shown in the experiments reported by this author.

The discrepancy observed here between specific activities measured *in vitro* and *in vivo* is as yet not fully explained. A similar disparity has previously been reported for rat adenohypophysial extracts when tested

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TABLE I. Corticotrophic Activity of Several Preparations of α-MSH.

Material tested	Melanophoretic activity, U/mg*	Corticotrophic activity in USP units/mg with confidence limits of assays for p = .05		
		In vitro	*In vivo*	
			Plasma B	Adrenal ascorbic acid
α-MSH #6	$3–5 \times 10^6$	1.8 (3.1– .4)		
α-MSH #6B (acid treated)	"	1.4 (2.4– .3)		
α-MSH #6A (alkali treated)	"	1.9 (6.1– .6)		
α-MSH #5D	"	1.7 (2.7–1.0)		
		1.2 (3.4– .5)		.11 (.30–.04)
" #14C	5×10^6	2.9 (10.8–1.4)	.12 (.13–.10)	.22 (.3 –.1)
" #21	1×10^7	3.5 (18.0–1.9)		
" #61B	"	3.6 (8.7–2.0)	.10 (.15–.04)	<.18

* *In vitro* assay of Shizume *et al.*(4). Melanophoretic activity expressed in units/mg.

against ACTH USP(11). The difference in activity was tentatively related to unequal rates of absorption by the adrenal tissue *in vitro* and unequal rates of inactivation and disappearance of the 2 substances *in vivo*(11, 12). Similarly the highly basic α-MSH could be tightly bound to blood or tissue proteins and rapidly become unavailable to the adrenal; or the acetyl-group on the N-terminus may reduce the affinity for the adrenal cortical tissue. It is also possible that (by enzymatically removing the acetylation of this N-terminus?) the adrenal tissue is capable of converting α-MSH to a more active corticotrophic molecule during the long contact *in vitro*.

In similar experiments, both *in vitro* and *in vivo*, no corticotrophic activity could be detected for β-MSH when it was tested in this laboratory against USP Reference Standard Corticotropin.

In view of the data presented here, it is suggested that the "active core" of the ACTH-molecule may be no larger than the tridecapeptide from the N-terminus.

Summary. Several preparations of highly purified α-MSH were studied for their adrenocorticotrophic activity *in vivo* and *in vitro* using USP Reference Corticotropin as a Standard. They showed corticotrophic activity by either assay procedure. β-MSH did not demonstrate similar activity. In view of data obtained, it is suggested that the "active core" of ACTH molecule may be no longer than the tridecapeptide from the N-terminus.

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Evidence for Non-Syncytial Nature of Cardiac Muscle from Impedance Measurements.* (25032)

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Until recently the spread of excitation in cardiac tissue was considered to be explicable in terms of protoplasmic continuity between cells. However, electron microscopy of cardiac muscle has shown that cellular membranes at the intercalated discs separate adjacent cells(1,2). If the myocardium is not a morphological syncytium, then some other basis for impulse transmission from cell to cell must be sought. Two possibilities are a) that the heart is a functional syncytium (*i.e.*, low resistance junctional membranes), or b) that some type of junctional transmission process is involved. In a previous study of frog heart with intracellular electrodes, ventricular cells were electrically isolated from their neighbors during perfusion with hypertonic solution(3). From these results it was concluded that the myocardium is not a functional syncytium. In the present study additional evidence is presented in support of this hypothesis. This evidence is based upon a comparison of the specific resistance of cardiac muscle with that of short-celled intestinal smooth muscle, and that of long-celled frog sartorius muscle. A syncytial structure would be expected to behave electrically like the long-celled tissue.

Methods. The D. C. resistances of 3 types of muscle were measured in the direction of fiber orientation: a) frog sartorius muscle, b) ganglion-free circular smooth muscle of cat intestine (*cf.* 4 for method of preparation), and c) ventricular muscle strips from dog and beef hearts. Relatively uninjured strips of ventricular trabeculae were excised from fresh beef hearts. Strips of parallel fibers were cut from papillary muscle of fresh dog heart. Each muscle strip was prepared and selected for uniformity of the transverse dimensions throughout the length of the strip. No samples thicker than 3 mm were used. The lengths varied from 15 to 30 mm, and weights from 15 to 150 mg. The muscle cross-sectional area (cm^2) was taken as average of one estimate obtained from direct caliper measurements of the transverse dimensions and one estimate from length and wet weight (uncorrected for specific gravity). D.C. resistances were measured with Wheatstone bridge technic using a cathode ray oscilloscope as the null-point detector. A Grass S-4 stimulator and stimulus isolation unit supplied rectangular pulses (3/sec., 30 msec. duration), and produced a voltage across the muscle of about 1 volt. A metal clip mounted on the micromanipulator served as one electrode and also supported one end of the muscle. A second electrode was immersed in reservoir filled with Tyrode's solution. The free end of the muscle was appropriately weighted and lowered into the reservoir in 4 steps. At each step, D.C. resistance and exposed length of muscle were measured. There was no evidence of polarization since polarity reversal did not alter the measured resistance. The resistance at each position was plotted against corresponding muscle length. From this linear plot, the slope or resistance per cm length of muscle was calculated. This slope was multiplied by the cross-sectional area to obtain the specific resistance in ohm-cm for a particular muscle strip. Resistance measurements were performed on each sample after soaking for about 2 hours in Tyrode's solution and again after soaking 2 hours in 10% Tyrode's made isotonic with sucrose. In some experiments the muscle was re-soaked in Tyrode's and a resistance value close to initial value was obtained. Tyrode's solution was used for mammalian tissues and frog Ringer's for frog sartorius. For mammalian muscles the 10% Tyrode's was made by mixing 9 volumes of 0.3 M sucrose with one volume of mammalian Tyrode's. The 10% frog Ringer's was made in a similar manner using 0.22 M sucrose. The volume of the soaking solution

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TABLE I. Mean Specific Resistances of Cardiac, Smooth, and Skeletal Muscles before and after Reduction of Interspace Ion Concentration.

Muscle	No. of samples	Specific resistance (ohm-cm)* Tyrode's	10% Tyrode's	Resistance ratio*	Interspace vol (ml/100 g muscle wt)	
Cardiac muscle						
Beef ventricular trabeculae	10	251 ± 17	1890 ± 116	7.6 ± .5	Cardiac	19† 30‡
Dog papillary strip	12	286 ± 13	1819 ± 85	6.4 ± .2		19§
Smooth muscle						
Cat intestine smooth muscle	20	118 ± 5	778 ± 42	6.7 ± .3	Smooth	28‖ 35¶
Skeletal muscle						
Frog sartorius	12	133 ± 11	367 ± 23	2.9 ± .2	Skeletal	35** 31†† 13‡‡

* Each value is mean ± stand. error of mean. Resistance ratio was calculated by dividing tissue resistance measured after soaking in 10% Tyrode's with tissue resistance measured after soaking in Tyrode's. † Sucrose space of cat ventricle *in situ*, calculated assuming 80% water content from data in(5). ‡ Cl or Na space of rabbit ventricle(6). § Histologically determined interspace of beef moderator band(7). ‖ Inulin space of frog stomach smooth muscle(8). ¶ Cl or Na space of rabbit small intestine(6). ** Sucrose, SO_4, and rapidly exchanging Na space of Ringer's soaked frog sartorius(9). †† Rapidly exchanging Na space of Ringer's soaked sartorius(10). ‡‡ Cl space with Donnan corrections(11).

was over 200 times the muscle volume and at least one replacement was made during the soaking period. All procedures were carried out at room temperature.

Results. The mean D.C. specific resistances of cardiac, (frog) skeletal, and intestinal smooth muscles were 270 ± 11, 133 ± 11, 118 ± 5, ohm-cm, respectively (Table I).‡ Ventricular muscle specific resistance was significantly higher ($p<.001$) than that of either skeletal or smooth muscle. Mean specific resistances after soaking for a minimum of 2 hr in 10% Tyrode's solution were 1838 ± 70, 367 ± 23, and 778 ± 42 ohm-cm, respectively for cardiac, skeletal and smooth muscles. Mean ratios of resistances in the 2 solutions for cardiac, skeletal and intestinal smooth muscles were 7.0 ± 0.3, 2.9 ± 0.2, and 6.7 ± 0.3, respectively. The resistance ratio of ventricular muscle was not significantly different from that of smooth muscle ($p>0.1$). However, the resistance ratio of skeletal muscle was significantly lower than that of either cardiac ($p<.001$) or smooth muscle ($p<0.01$). In other words the tissue resistances of cardiac and smooth muscles increased much more than did that of skeletal muscle when the interspace ion concentration was reduced 10-fold.

The large increase in ventricular muscle resistance after soaking in 10% Tyrode's is not attributable to a loss of intracellular potassium. Beef ventricular trabeculae immersed for 2 hours in 0.3 M sucrose lost 9 μeq potassium/g wet weight of muscle. This amount is less than 10% of the total muscle potassium and far from the loss necessary to account for the resistance increase found. The relatively small increase in sartorius muscle resistance after soaking in 10% Tyrode's could not be due to insufficient reduction of interspace ion concentration since duration of the soaking period employed was about 10 times the reported half-time for interspace sodium washout(12).

Discussion. The resistance increase following interspace ion depletion was found to be less in skeletal muscle than in cardiac or smooth muscles. The most probable explanation for this difference in resistance change is that a smaller proportion of the total current passed through the cells of cardiac or smooth muscles than those of skeletal muscle. Preferential current flow through the interspace in cardiac and smooth muscles could have been due to a large fraction of the total cross-sec-

‡ Specific resistances of beef and dog ventricular muscle were not significantly different and have been pooled for comparison with skeletal and smooth muscles.

tional area being interspace, or to a large resistance in current flow through the cells. If the whole tissue were interspace, all the current would of necessity pass through the interspace and the expected specific resistance would be that of Tyrode's (44 ohm-cm(4)). However, the reported values for the interspaces of cardiac, skeletal, and smooth muscles, although variable, are not sufficiently different to account for the differences in the resistance changes (Table I). Therefore, the current flow paths through the cells of cardiac and smooth muscles appear to be of high resistance. This high resistance is more likely due to the high resistance of cell membranes than to high myoplasmic resistance.

Other studies predict that the current flow path through smooth muscle cells should involve a succession of high resistance cellular membranes(13). Our results lead to the conclusion that this may also be true of cardiac muscle. This is consistent with the observation that ventricular muscle specific resistance is larger than that of skeletal muscle. It would appear that cardiac muscle cells are separated from each other by high resistance cellular membranes and do not form a low resistance functional syncytium.

Summary. The D.C. specific resistances of dog and beef ventricular muscle strips were compared with those of cat intestine circular smooth muscle, which is a non-syncytial tissue, and of frog sartorius muscle where individual cells extend almost the full length of muscle. Resistances were measured in the direction of fiber orientation before and after the interspace ion concentration was reduced by soaking in isotonic 10% Tyrode's-sucrose solution. Cardiac and smooth muscle resistances were much more sensitive to interspace ion depletion than was skeletal muscle resistance. It is suggested that the current flow path through the cells of cardiac muscle is of high resistance due to large number of high resistance cellular membranes involved, as in smooth muscle. It was concluded that cardiac muscle cells are separated from each other by high resistance membranes and do not form a functional syncytium.

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Infectious Ribonucleic Acid Derived from Enteroviruses.* (25033)

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In recent years the ribonucleic acid (RNA)-containing fraction of a number of viruses has been shown to be infectious. The evidence supports the premise that the active ingredient of this infectious portion is RNA; infectivity is inactivated by crystalline ribonuclease (RNAase) but not by gamma globulin prepared from specific antiserum or by desoxyribonuclease (DNAase)(1). In earlier ex-

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periments *in vivo* technics in plants, animals and chick embryos were used to demonstrate infectivity of RNA preparations made from tissues infected *in vivo*. Recently, Alexander *et al.*(1), using the phenol extraction method of Gierer and Schramm(2), demonstrated that under certain conditions of molarity and pH infectious RNA derived from polioviruses regularly produced plaques in tissue culture, and that the infectious capacity of the RNA was, under these conditions, proportional to dilution. A simple quantitative tool was then available for the study of infectious RNA. Most of their experiments were carried out using highly purified or partially purified poliovirus preparations. The work to be reported here demonstrates that their methods are equally applicable to crude tissue culture-grown virus suspensions, and can be successfully used to prepare infectious RNA from other members of the enterovirus group. RNA derived from Coxsackie A7, B4 and B5, and Echo types 1 and 8 induced plaques in which the intact virus supplying the RNA could be demonstrated. Previous exposure of the RNA-containing fraction to crystalline RNAase destroyed its infectivity, whereas DNAase had no significant effect.

Materials and methods. Virus strains used were 1) a heat-resistant mutant picked from a type 1 poliovirus population (Mahoney strain); 2) Coxsackie B5 virus grown from spinal fluid of a newborn infant with meningoencephalitis and probable myocarditis; 3) Coxsackie B4 virus from stool of patient with aseptic meningitis; 4) Echo 1 and Echo 8 strains obtained through courtesy of Dr. Heinz Eichenwald, N. Y. Hospital; and 5) Coxsackie A7 virus obtained from Dr. Karl Habel, Nat. Inst. of Health. The polio and Coxsackie B strains were grown in HeLa cells and amnion cells in Scherer's maintenance solution with 10% horse serum (MS) and were tested on amnion cells in monolayers in 60 mm Petri dishes. Echo viruses and Coxsackie A7 virus were grown in HeLa cells and tested in HeLa cell monolayers in Petri dishes. *Cell lines.* HeLa cells used were the L5 line grown from a clone isolated by Leidy *et al.* and picked for sensitivity to polioviruses. Amnion cells were the Cold Spring Harbor passage line of amnion cells (Fernandes(3)). HeLa cells were grown in Puck's modification of a Weymouth solution(4) and amnion cells in Eagle's solution with double the usual concentrations of amino acids and vitamins. *Monolayers.* Monolayers were prepared in 60 mm Petri dishes by adding approximately one million cells/plate in 4 ml of Puck's solution. Plates were incubated in an atmosphere of 5% CO_2 for 3 days, washed 2 $\times$ with a Tris-buffered nutritive solution to remove antibody, and inoculated with 0.1 ml of virus. After a 30-minute adsorption period a 1% agar overlay was added and the plates incubated in CO_2 for 4 days. At this time a neutral red-containing agar overlay was added and plaques were counted 3 to 4 hours later. If the inoculum was whole virus both wash solutions and virus diluent consisted of modified Earle's solution with lactalbumin, yeast, glucose, and Tris buffer added at pH of 7.2. If the inoculum was RNA, the second wash solution consisted of 0.65 M NaCl and 0.03 M Tris solution buffered to pH 7.6, and RNA dilutions were made in 0.9 M KCl plus Tris solution buffered to pH 8.0. Otherwise, RNA and whole virus were similarly treated. *RNA preparation.* The virus for RNA preparation was grown in HeLa or amnion cells in MS and harvested when 3^+ to 4^+ cytopathogenic action became evident. Suspensions were routinely frozen before use. When thawed, they were centrifuged at 1,500 rpm for 10 minutes and the supernatant used as the whole virus from which the RNA was derived. The phenol extraction technic for removing protein from RNA, noted briefly below, is described in detail by Alexander *et al.*(1). The procedure was carried out with chilled materials and equipment through the ether washing stage. In a centrifuge tube 2.5 ml of distilled or washed Mallinckrodt reagent phenol in water-saturated solution was mixed 1:1 with virus preparation which consisted of 2 ml of virus suspension, 0.3 ml of 5 M NaCl and 0.2 ml of phosphate buffer at pH 7.3. Virus and phenol were shaken by hand 4 minutes and separated by quick centrifugation at 12,000 rpm. The aqueous phase was removed, mixed 1:1 with saturated solution of phenol, shaken for 2 minutes and centrifuged. The third ex-

TABLE I. Comparison of Titer of Intact Enteroviruses and Their Infectious "RNA."

	Intact virus diluted 1:1 with				RNA diluted 4:1 with				RNA dilution		
	BSS*	RNAase	NMS†	Specific antiserum	Dilution‡	RNAase	DNAase	NMS	10^{-1}	10^{-2}	10^{-3}
Type 1 polio (4×10^{-6})§	41‖	35	70	0	40¶	0	56	0		17	0
	44	27	60	0	72	0	92	0		11	0
	50	46	47	0	88	0	41	0		10	0
	57	30	60	0	94	0	131	0			
T =	1.2×10^8	9×10^7	1.5×10^8		7.4×10^3		8×10^3			1.3×10^4	
ECHO 1 ($1 \times 10^{-4.5}$)	14	28	25	0	28**	0	14	0	18	0	0
	23	32	21	0	30	0	59	0	18	4	0
	10	23	23	0	66	0	62	0		4	
	22	30	17	0	16	0	56	0			
T =	5×10^6	1.4×10^7	7×10^6		7×10^2		1×10^3		1.8×10^3	3×10^3	
ECHO 8 (2×10^{-4})	6	2	23	0	5**	0	17	0	2	0	0
	11	6	15	0	11	0	16	0	1	0	0
	2	6	15	0	9	0	13	0		0	0
	1	1	4	0	24	0	18	0			
T =	2.5×10^5				2.4×10^2		3.2×10^2		2×10^2		
Coxsackie A-7 (2×10^{-4})	16	11	14		39**	0	3+	0	5		
	10	14	18		16+	0	22	0	1		
	18	14	20		22+	0	6+	0			
		11	17		12+	0	6+	0			
T =	7×10^5	6×10^5	7×10^5		4.4×10^2		1.8×10^2		3×10^2		
Coxsackie B-4 (2×10^{-4})	9	6	10	0	17**	0	24	0	7	0	0
	15	15	9	0	18	0	13	0	12	0	0
	14	8	17	0	11	0	19	0		0	0
	15	9	11	0	1	0	12	0			
T =	6×10^5	5×10^5	6×10^5		2.4×10^2		2.8×10^2		1×10^3		
Coxsackie B-5 (4×10^{-6})	72	63	95	0	50+¶	0	126	0		12	8
	97	64	91	0	88	0	102	0		18	2
	47	63	94	0	88	0	129	0		26	0
	77	62	99	0	99+	0	117	0			
T =	1.8×10^8	1.6×10^8	2.4×10^8		9×10^3		1.2×10^4			1.9×10^4	3×10^4

* Modified balanced salt solution. See *Materials and methods.*
† Normal monkey serum.
‡ 0.9 M KCl + Tris solution, pH 8.
§ Titer of intact virus inoculum.
‖ No. of plaques on monolayers.
¶ RNA diluted 1:9 initially.
** " " 1:1 " .

traction duplicated the second. This final aqueous phase was washed by shaking 5 × with more than equal quantities of cold ether to remove residual phenol, the ether being decanted and discarded. Residual ether was removed by bubbling nitrogen through final solution for 5 minutes. This final solution was diluted promptly 1:1 or 1:9 in 0.9 M KCl plus Tris at pH 8.0, since infectious RNA seems to be stable in such solutions(1). *Normal serum* was undiluted normal monkey serum free of specific antibody to the virus being tested. Specific antisera were monkey serum for poliovirus and rabbit serum obtained from Microbiological Associates for the Coxsackie B viruses and Echo viruses. Coxsackie A7 antiserum was not available. *Enzymes.* Crystallized ribonuclease of bovine origin was obtained from Worthington Biochemical Corp. and made to contain a final test dilution of 2 μg/ml in 0.9 M KCl Tris solution for RNA testing, and Tris-buffered isotonic balanced salt solution for whole virus. This variable was introduced because RNA titers are greatly reduced in isotonic solution and whole virus titers are somewhat reduced in hypertonic solutions. Desoxyribonuclease was obtained from Worthington Biochemical Corp. and made to contain final test solution of 5 μg/ml in 0.9 M KCl Tris solution with 0.003 M $MgCl_2$ added. *Tests.* In each instance, original whole virus preparation was titered on same day in same lot of plates with the same serum and enzyme dilutions as the RNA preparation. The single exception is the variable in the RNAase dilutions which were made in hypertonic solution for RNA testing and in isotonic solutions for whole virus testing.

Results. RNA-containing fractions of 6 enteroviruses were prepared from crude tissue culture virus suspensions by the phenol extraction method of Gierer and Schramm. The intact virus was compared simultaneously with its free RNA for their respective titers of plaque-forming agents in human cell monolayers. The intact virus titer was compared after exposure to salt solution, RNAase, normal serum and type-specific antiserum before seeding on monolayers, and in the same experiment the plaque titer of its RNA preparation was measured in the same lot of monolayers in the presence of hypertonic salt solution, RNAase, DNAase, and normal serum.

The Table is designed to point up the differences between whole virus and its RNA, and to show some of the reasons why the plaque-forming agent in RNA preparations is considered to be infectious ribonucleic acid and not small quantities of residual whole virus. Intact virus infectivity is not significantly affected by RNAase and normal serum, but is totally inactivated at the dilutions used by specific antiserum. RNA, on the other hand, is totally inactivated by RNAase and normal serum and not affected by DNAase. The RNAase content of our specific antisera made them unsatisfactory for testing their neutralization of the plaque-forming agent in the RNA preparation. All serum tested in our laboratory contained at least some RNAase, and RNA is routinely inactivated by normal or immune serum. If gamma globulin is prepared from immune serum this RNAase-free antibody will inactivate whole virus and not RNA(1). Gamma globulin was not used here, however.

In every instance reported here, plaques produced by RNA contained infectious particles which were unaffected by normal serum but were neutralized by antiserum specific for the intact virus from which the RNA was prepared. No antiserum for Coxsackie A7 was available, but the virus grew in the presence of normal serum. In brief, the infectious RNA entered host cells and directed the cells to produce type-specific whole virus. This provides additional evidence that the nucleic acid fraction carries some, if not all, of the genetic potentialities of the whole virus.

The data presented show further that the plaque titer of RNA was usually between 3 and 4 logs lower than that of whole virus, a differential which is not yet understood. It is of interest, however, that the differential is consistent throughout the series.

A simple, and probably generally applicable tool is now available for studies of virus RNA. Infectious RNA can be prepared with ordinary laboratory equipment from small quantities of crude tissue culture virus in less than one hour. The only requirements are that

there be at least 10,000 infectious particles present in the original virus suspension and that the virus be an RNA-containing one.

Several attempts have been made to produce infectious nucleic acid by the same method from vaccinia, a presumed DNA-containing virus. So far all such attempts have been unsuccessful despite the fact that some were carried out in parallel with successful enterovirus experiments, using similar quantities of virus and the same materials throughout.

Summary. 1) RNA-containing fractions have been prepared from crude tissue culture virus suspensions of 6 varieties of enterovirus by the method of Gierer and Schramm. Each RNA preparation has produced plaques on human cell monolayers. The plaque-forming agent is completely inactivated by RNAase but not by DNAase. RNA is, therefore, an essential component for the infectivity of the preparation. 2) The plaques formed by RNA fraction contain intact virus from which RNA is prepared. The RNA directs the cell to replicate not only the specific RNA, but also a new highly specific protein needed for synthesis of intact original virus.

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Assay and Properties of Serum Inhibitor of C'1-Esterase* (25034)

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Evidence has been presented that the first component of human and guinea pig complement (C'1) exists in serum as a proenzyme which may be activated by antigen-antibody complexes(1-5). Human C'1 was also activated by plasmin(1,6) and, in a partially purified state, by autocatalysis(6,7). Two activities were demonstrable for human "activated C'1" (C'1-esterase): hydrolysis of certain synthetic amino acid esters, of which N-acetyl-L-tyrosine ethyl ester (ALTEe) was most susceptible(6-8); and inactivation of the second and fourth components (C'2 and C'4) of human complement(3,7). Both activation of C'1 and enzymatic activity of C'1-esterase were inhibited by a property of fresh human serum which was non-dialyzable, heat-labile (56°-30 min.), and unrelated to any of the 4 recognized components of complement(6,8). However, the precise relationship of the serum inhibitor of activation and of esterolysis could not be defined in the absence of purification studies. The present investigation was undertaken to provide information for subsequent purification of the serum inhibitor of C'1-esterase. The assay and properties of this inhibitor in normal serum are described and a comparison is given of levels of inhibitor in various mammalian sera.

Materials and methods. C'1-esterase—Partially purified human C'1 was prepared by published procedures(7) and activated autocatalytically by adjustment of physico-chemical conditions (pH 7.4, ionic strength 0.15, 37°, 15 min)(6,7). Rabbit C'1 and C'1-esterase were prepared from normal rabbit serum by identical procedures. *ALTEe*—N-acetyl-L-tyrosine ethyl ester, synthesized in the Dept. of Chemistry of Western Reserve Univ., was used as a substrate for C'1-esterase. The ester was dissolved in 2-methoxyethanol (methyl cellosolve) to a stock concen-

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tration of 1.6 M. *Mammalian Sera.* Blood was drawn without anticoagulant, clotted at room temperature for about 1 hr, and held overnight at 1-5°. Serum was separated by centrifugation in the cold and either maintained at 0° for use within 24 hr or frozen at −45°. Blood was obtained from healthy human donors by venipuncture; from rats and guinea pigs by exsanguination from the carotid artery; and from mice, rabbits and monkeys by cardiac puncture. Mouse serum represented a pool from 16 CF-1, female white mice anesthetized intraperitoneally with Nembutal® (25-35 mg/kg) and bled by cardiac puncture after thoracotomy. Monkey (rhesus) serum was obtained through the courtesy of Dr. F. C. Robbins, Cleveland Metropolitan General Hospital, and bovine, porcine, and ovine sera through the courtesy of Swift and Co., Cleveland. *Assay of C'1-esterase* (6,8). Esterase activity was measured by microformol titration of the acid liberated from ALTEe during 15 min. incubation with enzyme at 37°, pH 7.4, ionic strength 0.15, and a final substrate concentration of 0.08 M. Typically, 1.88 ml of pH 7.4 phosphate buffer of ionic strength 0.15 and 0.5 ml of an appropriate dilution of esterase were preincubated at 37° and 0.125 ml of ALTEe at 37° was then added. One ml aliquots were withdrawn at 0 time and at 15 min. and added immediately to one ml of neutralized (phenolphthalein) 37% formaldehyde. Each sample was microtitrated with a one ml microsyringe (Micrometric Instrument Co., Cleveland) using 0.05 N NaOH and one drop of 1% alcoholic phenolphthalein as indicator. Esterase activity was expressed in units based on the difference between the 15 min. and 0 time titrations. One unit was defined as that amount of enzyme which hydrolyzed an amount of ALTEe equivalent to a net titration of 0.01 ml of 0.05 N NaOH.

Results. 1) Assay of serum inhibitor of C'1-esterase. The studies to be described were confined entirely to inhibition of esterolytic activity of C'1-esterase. Accordingly, the assay for serum inhibitor was a modification of the assay for esterase activity(8). Various volumes of human serum, taken to 0.5 ml with 0.15 M NaCl, were preincubated

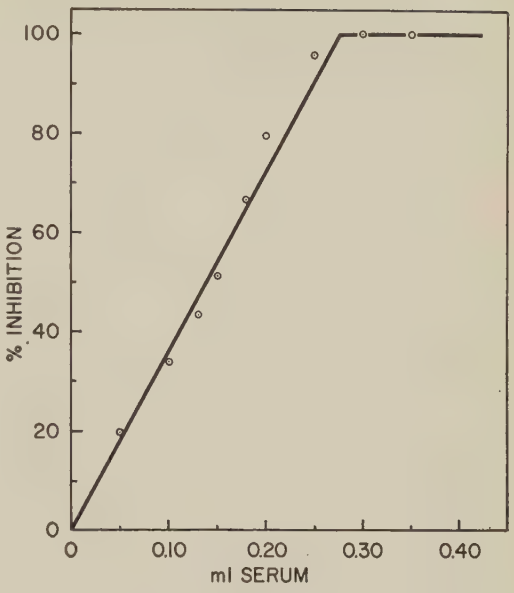

FIG. 1. Proportionality curve for inhibition of human C' 1-esterase (38 units/ml) by normal human serum.

at 37° with a constant amount of human C'1-esterase and phosphate buffer at a final pH of 7.4 ± 0.4 and ionic strength of 0.15. ALTEe was added, the amount of residual free enzyme measured by microformol titration, and the amount of esterase inhibited calculated. The assay was found to be linear over the entire range of inhibition (Fig. 1). The effect of varying length of preincubation time of esterase and inhibitor was studied by reacting enzyme, buffer, and serum at 37° in the absence of ALTEe. At intervals of 1, 10, 20, 30, 60, and 120 min., aliquots were withdrawn, substrate added, and esterase inhibition determined. Maximal inhibition was found within 1 min. and was unaffected by further incubation(8). The effect of varying the concentration of esterase was also studied. In every case, a given amount of serum inhibited the same *number* of units of esterase regardless of initial concentration of enzyme, indicating that inhibition was stoichiometric (Table I). One unit of inhibitor was defined as that volume of serum which inhibited 10 units of esterase, as measured by micro-formol titration.

On the basis of these studies, the following assay procedure was adopted: 1.38 ml of pH

TABLE I. Stoichiometry of Interaction of Serum Inhibitor with C' 1-Esterase: Various Concentrations of Esterase with Constant Serum Inhibitor.*

Esterase added, units/ml	Esterase inhibited, units/ml
16.4	11.4
23.8	10.8
32.8	10.7
36.9	10.8
41.0	12.6

* 0.1 ml of human serum, taken to 0.5 ml with 0.15 M NaCl.

7.4 phosphate buffer at ionic strength 0.15 was preincubated at 37° with 0.5 ml of human C'1-esterase at a concentration of 40 ± 2 units/ml and various volumes of the serum to be assayed for inhibitor, taken to 0.5 ml with 0.15 M NaCl. After 5-10 min., 0.125 ml of 1.6 M ALTEe at 37° was added, the mixtures were incubated at 37° for 15 min., and the residual free enzyme was determined by titration in the usual manner. Number of units of inhibitor in the unknown serum could then be calculated. For example, if 0.25 ml of serum inhibited 15 units of enzyme, the serum contained 1.5 units of inhibitor in 0.25 ml or 6 units/ml. Since the enzyme-inhibitor interaction was stoichiometric, inhibitor levels could be determined at any point on the inhibition curve. An enzyme control in the presence of substrate and absence of inhibitor was always included to reestablish the potency of the enzyme.

The precision of the assay was determined by 10 measurements of the inhibitory activity of a single human serum at each of 3 concentrations (0.10, 0.15 and 0.20 ml of serum). The mean of the 30 determinations was 7.2 units/ml with a range of 6.3-8.1 and a standard deviation of ± 0.42. *2) Some properties of serum inhibitor of C'1-Esterase.* a) *Time-temperature stability.* The inhibitor in human serum was stable for at least 24 hr at 0°, 21° and 37° and for at least 4 months at −45°. It was unaffected by freezing and thawing 2 times. During incubation for 30 min, 20% of the inhibitor was inactivated at 52°, 90% at 56°, and 100% at 60°. b) *pH stability.* Aliquots of human serum were adjusted to various pH values with 0.15 N HCl or 0.15 N NaOH, brought to constant volume with 0.15 M NaCl, and allowed to stand overnight at 0°. Each sample was then readjusted to neutrality and assayed. The inhibitor was stable under these conditions between pH 6 and 10 but progressive inactivation occurred at pH values below 6. Complete inactivation was observed at pH 3.6; 65% inactivation at pH 4.8. c) *Effect of alcohols and ammonium sulfate.* The inhibitor was increasingly labile to methanol concentrations greater than 15% at −5°. Similarly, as reported previously(8), the inhibitor did not survive fractionation of human serum with ethanol by procedures of Cohn *et al.*(9). However, essentially all of the inhibitory activity of human serum could be recovered from the supernatant fraction resulting from precipitation with 40% ammonium sulfate. 3) *Comparative levels of serum inhibitor of C'1-Esterase in various mammalian sera.* Serum from 4 of the 9 species tested contained demonstrable inhibitor of human C'1-esterase (Table II). Human, monkey and guinea pig sera had comparable and relatively high titers of inhibitor, while rabbit serum was only about one-third as active. Rat and mouse sera contained spontaneous esterase activity and therefore no measurable inhibitor. Bovine, porcine and ovine sera demonstrated neither spontaneous esterase nor inhibitory activity. The inhibitor in rabbit and guinea pig serum had the same thermal lability as in human serum. Monkey serum was not tested.

Since human C'1-esterase was used in these experiments, the possibility existed that the variation in inhibitor levels among different species was a reflection of species specificity. A partial answer to this problem was provided by preparing and activating partially purified

TABLE II. Serum Inhibitor of Human C' 1-Esterase in Various Mammalian Sera.

Serum	Avg inhibitor titer, units/ml
Human (6 pools of 25)	6.4 (5.6-7.2)
Monkey (2)	5.0
Guinea pig (3)	5.2
Rabbit (3)	1.8
Rat (3)	0
Mouse (pool of 16)	0
Bovine (1)	0
Porcine (1)	0
Ovine (1)	0

TABLE III. Species Specificity of Rabbit and Human Sera as Sources of Inhibitor *vs* C′ 1-Esterase Prepared from Rabbit or Human Serum.*

Inhibitor source	C′ 1-Esterase source	Inhibitor titer, units/ml
Rabbit serum	Rabbit	2.0
" "	Human	3.0
Human "	Rabbit	5.0
" "	Human	7.7

* Each serum was assayed against equivalent amounts of rabbit and human C′ 1-esterase under the usual conditions.

rabbit C′1 by the same procedure used for human C′1(6,7). The inhibitory activity of rabbit and human sera *vs.* rabbit and human C′1-esterase was then compared (Table III). Rabbit serum was again found to have a lower titer of inhibitor than human serum even when tested against rabbit esterase. It was concluded, therefore, that at least with respect to these two species, no marked species specificity existed in the interaction of C′1-esterase and serum inhibitor.

Discussion and Summary. An assay is described for measurement of a serum inhibitor of an esterase derived from preparations of the first component of complement (C′1-esterase). Esterolysis of N-acetyl-L-tyrosine ethyl ester by C′1-esterase is inhibited instantaneously and stoichiometrically by fresh human serum. Units of C′1-esterase and of serum inhibitor are defined. The inhibitor in human serum is stable at –45° for at least 4 months, at 37° for at least 24 hr, and at 48° for at least 30 min. It is completely inactivated during 30 min. incubation at 60° and is labile at 0° at pH values below 6. The inhibitor is inactivated at –5° in the presence of methanol concentrations of 15% or greater but is recovered quantitatively in a 40% ammonium sulfate supernatant fraction. These data are being applied to purification of the inhibitor from human serum. Purification procedures and properties of the purified inhibitor will be reported elsewhere.

Monkey and guinea pig sera inhibit human C′1-esterase to about the same extent as human serum, while rabbit serum is about one-third as active. No marked species specificity exists in the interaction of inhibitor in human or rabbit serum with human or rabbit C′1-esterase. These observations are being employed on possible role of inhibitor in experimental hypersensitivity.

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Relationship of Infectious Canine Hepatitis Virus to Human Adenovirus (25035)

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A virus strain (Utrecht) was isolated from a fatal case of hepatitis contagiosa canis (HCC) in trypsinized dog kidney cell culture. Inclusions as described by Leader(1) were found in nuclei of these cells when stained with hematoxylin and eosin. In cross complement-fixation tests with a reference dog serum* and a reference virus strain,† the iso-

* Obtained from Dr. H. Kunst, originally received from Prof. Hjarre (Stockholm).

† Received through courtesy of Dr. J. H. Gillespie, Cornell University, Ithaca, N. Y.

lated virus was identified as a strain of hepatitis contagiosa canis (HCC) virus. A microscopic comparison of the intranuclear inclusions in dog kidney cells and similar inclusions found in adenovirus infections of our human epithelial cell lines revealed so striking a resemblance that further investigation into the relationship of the Utrecht or other HCC virus strains and the adenovirus group seemed indicated. The characteristics of the adenovirus group(2) are: 1. Cytopathogenicity for susceptible tissue culture cells. 2. Nonpathogenicity for the usual laboratory animals. 3. A complement-fixing antigen common to all members. 4. Resistance to ethyl ether. HCC virus was tested regarding these 4 points.

Materials and methods. Virus. Two strains of HCC virus were obtained, one tissue culture adapted† and the other as virulent dog liver.‡ Strain Utrecht was isolated in dog kidney cell culture in our laboratory. *Tissue culture.* Trypsin dispersed dog kidney cells were prepared according to a modification of the method described by Youngner(3) and grown in a medium consisting of Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate and 5% calf serum. Cells were maintained in medium 199 with 5% calf serum. Sometimes second passage cells were used. T_1-cells(4) were grown and maintained in the same media. *Complement fixation.* Quantitative complement-fixation tests were performed in plates. Two hemolytic units of complement were used. The mixtures were incubated overnight at 4°C.

Results. 1. The cytopathogenic effect of HCC virus strain Utrecht in dog kidney cells consists in a rounding of cells, which become highly refractile. Sometimes characteristic grapelike clusters of rounded cells are formed. Finally the cells detach from the glass. With the adapted strain, the CPE of undiluted virus usually is completed within 2 to 3 days, whereas the incubation period increases with dilution of the infecting virus. After fixation with Bouin's fluid and staining with hematoxylin and eosin, the first changes in the nuclei seem to be one or more small finely granular eosinophilic masses surrounded by a clear zone. More nuclei contain one large granular eosinophilic mass, separated from the nuclear membrane by a clear zone. Often this zone is subdivided into compartments by radiating lines. The nucleoli are pushed towards the nuclear membrane or incorporated in the central mass. In the latter case they are surrounded by a clear halo. In later stages they are no longer recognizable. Sometimes the affected nuclei are enlarged.

These findings agree exactly with those described by Boyer(5) for adenovirus type 3 and 4 in HeLa cells and found with our adenovirus strains of type 3, 4 and 7 in the human kidney cell line T_1. The only difference is that HCC virus seems not to produce crystal-like structures.

2. As is known from experimental evidence (6,7) the HCC virus is not infectious for guinea pigs, rats, mice, baby mice, rabbits and chick embryos. In our experience, the Utrecht strain is somewhat toxic for suckling mice; we could not pass the virus, nor could we find histological reactions indicating an infection. The chorioallantoic of 10-day-old duck embryos was not susceptible either. The only naturally susceptible animals seem to be dogs and foxes(8).

3. In preliminary experiment, an antigen was prepared from virulent dog liver according to directions of Mansi(9). Human sera were selected regarding presence or absence of complement-fixing antibodies for adenovirus antigen. The antigen we used is made from adenovirus type 1 and 5 grown in T_1 cells. The results are shown in Table I. All sera positive with adenovirus antigen react with HCC liver antigen too.

After these promising results, antigens were made from 3 strains grown in dog kidney cell culture. Strain Rotterdam was received as a virulent dog liver suspension and passed once in tissue culture; strain Ithaca was received in its 119th dog kidney passage. The antigens were heated for one hour at 56°C. Dr. van der Veen (Tilburg) tested these antigens with paired sera from patients with adenovirus infections in complement fixation. The type isolated from their throat washings is indicated

‡ Received from Dr. O. Bosgra, Philips-Roxane, Weesp, and Dr. G. M. van Waveren, Rijksserum Inrichting, Rotterdam.

TABLE I. Comparison of C. F. Titers in Human Sera Tested with Adenovirus and HCC Liver Antigen.

Serum No.	Complement fixation titer: Adenovirus antigen	HCC liver antigen
902	32	8
943	128	32
964	32	8
970	32	8
985	64	32
930	<4	<4
944	<4	<4
951	<4	<4
963	<4	<4
1000	<4	<4

Titers expressed as reciprocal of highest dilution giving complete fixation.

in the Table. Human control sera from cases of nonadenovirus infections were included. Table II shows the results of complement fixation tests carried out with HCC antigens and the adenovirus antigen made from adenovirus type 4 grown in HeLa cells. All sera show an almost equal rise in complement-fixing antibodies, whether they were tested with the known adenovirus antigen or each of the HCC antigens. As the sera were derived from patients infected with various adenovirus types, the HCC strains and the adenovirus types share a common C.F. antigen.

4. After treatment with 20% ethyl ether during 18 hours at 4°C, a virus suspension of strain Utrecht had the same infectious titer as the untreated control (10^{-4}).

Discussion. From these results, it seems justified to consider the virus of infectious canine hepatitis as a member of the adenovirus group.

In some of the clinical manifestations, infectious canine hepatitis also resembles adenovirus infections in man. Many exposed dogs suffer from conjunctivitis, fiery redness of the mucous membranes of the oral cavity and tonsillitis. Many show a transient corneal opacity of one or both eyes(10,11). A similar transient keratoconjunctivitis is described for adenovirus type 3(12). A keratoconjunctivitis of longer duration is observed in persons infected with adenovirus type 8(13). The association of adenoviruses with pharyngoconjunctival fever is well known(2). On the other hand, symptoms of the respiratory system are rare in dogs with infectious hepatitis. Adenoviruses in man do not show a tropism for endothelial and liver cells, as is characteristic for Rubarth's disease(10) nor is a human liver disease known with similar intranuclear inclusions. Lethality of human adenovirus infections is extremely low.

With respect to its tendency for persistence

TABLE II. Complement Fixation Titer of Sera from Patients with Adenovirus and Other Infections.

Serum patient No.		Isolated type adenovirus	Antigens: Adenovirus	Hepatitis contagiosa canis: Utrecht	Rotterdam	Ithaca	Control dog kidney
1	A*	3	5	5	10	5	0†
	C		20	40	40	40	0
2	A	4	5	10	10	10	ND
	C		40	40	80	40	0
3	A	4	10	10	10	5	0
	C		1280	320	640	1280	0
4	A	7	0	5	5	5	0
	C		40	40	40	40	0
5	A	14	10	10	10	20	0
	C		80	40	80	80	0
6	A	Control	0	0	0	0	0
	C		0	0	0	0	0
7	A	Control	0	10	10	10	0
	C	(Influenza A)	0	10	10	10	0

* A = acute phase serum. C = convalescent phase serum.
† 0 = <1:5; ND = not done.
Titers expressed as reciprocal of highest dilution giving complete fixation.
Test by Dr. J. van der Veen.

after infection, HCC virus behaves as a member of the adenovirus group. The virus can be excreted with the urine for as long as 271 days(11). The virus was "unmasked" in noninoculated dog kidney cell cultures derived from a dog 3 months after recovery from an infection(14). In the same way, adenovirus was detected in cultures made from human adenoid tissue.

As far as we know, HCC virus is not pathogenic for man. The claim of inapparent human infections made by some authors(15) was based on the finding of C.F. antibodies in human sera. This should be reviewed in the light of the relationship between HCC strains and adenoviruses.

It is not yet known if the virus of infectious canine hepatitis is a new member of the adenovirus group or related to one of the human or simian types. Dr. van der Veen informed us that the prototype adenovirus strains of types 1-17 could not be neutralized by our guinea pig antiserum to the Utrecht strain of HCC virus at a serum dilution of 1:4. This serum had a neutralizing titer of 1:320 for the homologous strain. Typing experiments are in progress and will be published. The host range and virulence of HCC virus points to a separate position among the members of the adenovirus group. Future experience will show whether the animal host range is appropriate for the further division of adenoviruses into human, simian and canine subgroups.

Summary. 1) The virus of infectious canine hepatitis was shown to share the 4 main properties of the adenovirus group, including common complement-fixing antigen. 2) The relationship between the disease of the dog and human adenovirus infections is discussed.

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Human Blood Group Substance B and *Escherichia coli* 086. (25036)

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Many investigators have considered selective influences of ABO blood groups on disease conditions. A marked selection against heterozygous fetuses and infants occurs, for example, when the mother is group O and the father is group A or group B(1). Moreover, Race and Sanger(2) have postulated that it is unlikely that the relationship between blood groups and disease would work only through maternal-foetal incompatibilities. A significant association has been found between the ABO groups and duodenal ulcer, carcinoma of stomach, and diabetes mellitus(3). Also, in infectious diseases, individuals of blood group B appear to possess a slightly greater degree of natural resistance to poliomyelitis(4).

McConnell(3) postulated that possibly these associations are merely ethnological, that patients with some diseases are of a racial group with high susceptibility to the disease and that their blood group frequencies are different from the rest of the population. Manuila (5) has also pointed out that natural variation in frequency of blood groups in a limited area, sampling errors, and technical errors may account for assumed association. Studies by Oliver-Gonzalez(6) indicated presence of serologic blood group active polysaccharides in animal parasites and Springer(7) has shown the presence of serologic blood group active substances in animals (gastric mucin of the horse and Castle's intrinsic factor from the hog), plants, several species of the enteric bacteria, and *Pseudomonas aeruginosa.* Springer also indicated that decomplemented high titered human anti-B sera temporarily inhibited growth of *Escherichia coli* 086. These findings suggested a new approach to the possibility of an association between blood groups and infectious disease. Of particular interest was the high group B activity in *Escherichia coli* 086, one of the *E. coli* serotypes associated with epidemic diarrhea of the newborn and the disproportionately high group A activity of the Vi antigen. Significance of the Vi antigen in immunization and protection of mice against challenge with virulent *Salmonella typhosa* is well documented (8), although its role, if any, in human infection is not clear.

Results. It seemed possible that relative susceptibility of different individuals to these and other microbial organisms and their products possessing blood group activity might be influenced by the individuals' blood group. Accordingly, a large number of bacteria, viruses, and toxoids were tested for serologic blood group activity by the hemagglutination-inhibition technic. The bacteria were cultured on meat extract agar plates containing no detectable blood group activity, suspended in saline and adjusted optically to standard density to contain about 2.4×10^9 organisms/ml. Serial dilutions in duplicate of the untreated and heat-killed suspensions of bacteria and of other materials in saline were prepared. Equal volumes of Anti-A and anti-B containing 4 units (4 times minimal quantity of Anti-A or anti-B required for agglutination of a standard suspension of appropriate erythrocytes) were added to each dilution series. Whenever inhibitory activity was found, it was greater with the heat-killed suspension. Purified Vi antigen(9) was consistently reactive for group A activity; solutions containing 6.25 μg/ml of the antigen sufficed for its detection. Slight A activity which was not consistently reproducible was obtained with several Vi-containing organisms (*S. typhosa* strain Ty2, *S. typhosa* strain ViI, *Paracolobactrum ballerup, E. coli* 5396/38) and *Shigella flexneri* 2a. Group B activity was detected only in 5 strains of *E. coli,* all of serotype 086:B7. In each of these strains, approximately 2.4×10^6 organisms sufficed for detectable group B activity. The other organisms tested included 23 serotypes of *E. coli, Salmonella paratyphosa* A, 10 different *Shigella* organisms, an Ogawa and Inaba strain of *Vibrio cholera, Staphylococcus pyogenes, Staphylococcus aureus, Corynebacterium xerose,* and 3 *Leptospira* organisms. Also tested were complement fixation antigens for typhus fever, lymphogranuloma venereum, Western equine encephalomyelitis, Eastern equine encephalomyelitis, mumps, herpes, and lymphocytic chorio-meningitis; poliomyelitis vaccine; diphtheria and tetanus toxoids. None of these organisms or materials reacted.

These findings raised 2 questions relating to antibody formation and resistance to infection with microbes possessing blood group active substances. In the first place, since animals do not respond ordinarily to their own antigenic substances, would the presence of a blood group active substance in a microbe or the microbe's products result in diminished antibody formation in infected individuals of the same blood group? The implied detrimental effect of such immunological response to one's own antigens was dramatized by Ehrlich with the expression "horror autotoxicus." The sera of 204 individuals injected with purified Vi antigen were assayed for Vi hemagglutinin(10) 14 days after injection. Although the geometric mean titer of individuals of Group A and AB was 95 and that of the others was 109, the difference was not significant. These findings may reflect the low level of blood group A activity in Vi

TABLE I. Response of a Rabbit to 4 Intravenous Injections of 1 ml of Washed 30% Human Group B Erythrocytes. Sera were absorbed with group O erythrocytes before testing for hemagglutinins to remove red cell antibodies other than anti-A or anti-B.

Serum	Hemagglutination titers		Bactericidal antibody titers	
	Anti-A	Anti-B	*E. coli* 086	*E. coli* 02
1st day—preimmunization	16	4	2.5	2.5
21st day, 12 days after last inj.—post-immunization	256	256	152.0*	2.5

* Absorption of 21st day serum with group B erythrocytes reduced bactericidal antibody titer against *E. coli* 086 to less than 2.5. Absorption of this serum with heat-killed *E. coli* 086 reduced its bactericidal titer to less than 2.5 and its anti-B titer to 4.

antigen, but are suggestive that ordinarily blood group activity in a microbe would not influence its antigenicity in the human host. Moreover, chimpanzees, which do not respond as well as man to purified Vi antigen (personal communication from Dr. G. Edsall and Major S. Gaines), are predominantly of blood group A(11), but whether this is merely a coincidental rather than a causal relationship is conjectural.

The other question was whether the reactivity of the isoantibodies against organisms possessing blood group activity would contribute to the body's defenses against these organisms. Bactericidal activity of pools of fresh normal human sera(12) against several strains of *E. coli* 086 did not reveal significant differences, however, between sera of individuals of different blood groups. On the other hand, the sera of individuals with elevated anti-B levels were considerably more reactive against *E. coli* 086 than control sera. Mean serum bactericidal antibody titer determined by quantitative photometric assay (13) of 5 mothers of group O who gave birth to group B babies and who were presumably stimulated with group B substance(14) was 6.3. In contrast, sera of 3 mothers obtained after homospecific pregnancies gave a mean titer of 2.7. In addition, in an individual injected subcutaneously with 0.2 ml of blood group specific substance B (obtained from gastric mucosa of horses, product of Knickerbocker Blood Bank, N. Y.) the bactericidal antibody titers against *E. coli* 086 rose from 2.0 to 17.9 or 9-fold; anti-B agglutinin titer rose from 32 to 16,384 or 512 fold. A slight rise in bactericidal antibody titer from 2.2 to 4.3 was noted also against an *E. coli* 02 strain which does not contain detectable blood group substance B.

To elucidate further the relationship between blood group B substance and *E. coli* 086, rabbits were injected with blood group B substance, human group B erythrocytes, heat killed *E. coli* 086, and living *E. coli* 086. The rabbit injected with blood group B substance showed no detectable anti-B hemagglutinin response although the bactericidal antibody titer of its serum against *E. coli* 086 rose slightly from less than one to 2.5. The rabbit injected with human group B erythrocytes responded much better (Table I). The simultaneous rise in anti-A has also been noted by other investigators(15), but rabbit anti-A had no enhanced bactericidal activity against *E. coli* 086. The results obtained with sera of rabbits injected with *E. coli* 086 are outlined in Table II. Antisera to 4 other species of Enterobacteriacea prepared by injections of organisms grown similarly to *E. coli* 086 showed no anti-B response, and therefore, the meat extract agar as a possible source of group B substance in *E. coli* 086 was ruled out. The relatively slight loss in bactericidal activity against *E. coli* 086 as a result of absorption of anti-B from *E. coli* 086 antisera indicated that although the B reactive grouping of *E. coli* 086 is a susceptible target for bactericidal reaction, it is relatively insignificant in the presence of an antiserum directed against the entire antigenic mosaic of the organism.

Summary. Blood group A activity was demonstrated in purified Vi antigen preparations and blood group B activity in whole cells of *Escherichia coli* 086. The immunological implications of these findings were investigated. The antigenicity of Vi antigen was essentially as great in group A or AB individuals as in those of other groups. Anti-B exerted a bactericidal effect upon *E. coli* 086, but further studies are obviously needed

TABLE II. Response of Rabbit #1 to Heat-Killed *E. coli* 086 and of Rabbit #2 to Viable *E. coli* 086. Rabbits were injected intravenously on day 1, 5, 9 and bled on day 1 and on day 21.

Sera—rabbit #1	Anti-B hemagg. titer	*E. coli* 086 agg. titer with heat-killed organisms	Bactericidal antibody titer *E. coli* 086	*E. coli* 02
1st day—pre-immunization	2	64	2.5	2.5
21st day—post-immunization	1024	1024	1960	2.5
Idem, after absorption with group B cells	2	1024	1560	2.5
Sera—rabbit #2				
1st day—pre-immunization	16	16	2.5	2.5
21st day—post-immunization	256	1024	666	2.5
Idem, after absorption with group B cells	2	1024	600	2.5

to determine the protective effect, if any, of anti-B against *E. coli* 086 or, indeed, of either isoantibody against any microbial agent which may possess blood group antigens. Nevertheless, cross-reactivity between blood group substance B and *E. coli* 086 affords a model for the possible influence of blood groups in resistance to infection against those microbial agents which may possess blood group antigens.

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Pyridoxine Deficiency in Congestive Heart Failure. (25037)

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Our previous studies have shown the occurrence of thiamine deficiency in patients with advanced congestive heart failure. The deficiency was demonstrated by the thiamine test dose(1), pyruvic acid levels in the blood (2), and determination of thiamine and cocarboxylase content in myocardial tissues of patients dying of congestive heart failure(3). In view of the fact that Vit. B_6 is a member of the Vit. B complex and is required for a great variety of enzymatic reactions, it appeared desirable to investigate Vit. B_6 metabolism in congestive heart failure.

Methods and material. The presence of Vit. B_6 complex is required for metabolic breakdown of tryptophan. In induced Vit.

B_6 deficiency, derangement of tryptophan metabolism is manifested by increased excretion of xanthurenic acid after a tryptophan load(4,5). Administration of Vit. B_6 corrects this aberration and xanthurenic acid excretion is greatly decreased or becomes normal(6). We have used the increased excretion of xanthurenic acid (XA) after a load test of tryptophan and its correction by administration of pyridoxine as an index of a relative pyridoxine deficiency. Twenty patients (14 males and 6 females) were selected from the medical services of the Hahnemann Hospital. The patients were hospitalized for treatment of congestive heart failure. The causes leading to heart failure were hypertension, arteriosclerotic coronary artery disease and rheumatic heart disease. No patient with heart failure was studied unless organic heart disease was clinically demonstrable and objective evidence of peripheral or pulmonary edema, tachycardia, tachypnea were present. Elevation in venous pressure and prolonged arm-to-tongue circulation time (Decholin) were demonstrated in each patient by the standard technics. *Controls.* Seventeen patients free from congestive failure served as controls. They were patients of similar age and sex distribution as the cardiac group (13 males and 4 females), hospitalized for treatment for chronic osteomyelitis, anxiety neurosis, cerebral thrombosis, fracture of the femur, phlebothrombosis and malignancy of gall bladder. The daily diet of both groups of patients was the standard Hahnemann Hospital 1.0 g sodium diet containing on the average 2900 calories with protein, carbohydrate and fat and vitamin content in excess of that recommended by NRC (Food and Nutrition Board). Every effort was made to encourage patients under study to consume the daily allowed food. However, it is possible that in a few instances, a portion of the food was not eaten. We are aware that this factor may contribute to variation in amounts of xanthurenic acid excretion. However, the validity of the results of the overall study are not impaired since our aim is to evaluate the status of Vit. B_6 nutrition in patients with congestive heart failure as they are observed in a general hospital under ordinary hospital care. The food intake of our patients represents probably the usual dietary experiences of hospitalized cardiac patients. None of the patients in our series, to our knowledge, were taking supplementary vitamins prior to the study. Xanthurenic acid excretion was determined by a modified method of Rosen(7) on a 24 hour urine specimen. A control XA excretion before the tryptophan load test was done. On the morning of the second day, a 10 g dose of d, l-tryptophan (Dow Chemical Co.) was given mixed in fruit juice and a second 24-hour urine collection obtained. On the morning of the third day, another 10 g dose of tryptophan was given as on the previous day and the third 24-hour urine was collected. On the morning of the fourth day, 50 mg of pyridoxine-HCl (U.S.P.) was administered intramuscularly 30 minutes before a third dose of 10 g of tryptophan was given, and this was followed by the fourth and final 24-hour urine collection. Urine specimens were collected on the ward under toluene, the reaction was adjusted to pH 5.5 with 5 normal hydrochloric acid, aliquots were stored in amber glass bottles at 4°C. Initially, all urine was refrigerated as soon as it was voided, but this was not continued since it was found that no significant loss of XA occurred when urine was maintained at room temperature. Accuracy of collections was checked by carrying out urinary creatinine determinations by the method of Folin(8). More accurate collections were obtained if collection bottles were kept at the bedside or carried with the patients to X-ray or other hospital locations during the study. When a variation in creatinine of more than 25% was found, the patient was dropped from the study. Complete collections were deemed essential, since preliminary studies had revealed that the largest amounts of xanthurenic acid excretion in both controls and patients with heart failure appeared in the first 8 hours after oral ingestion of tryptophan. During the period of study, all patients were given supplements of 10 mg of thiamine and 5 mg of riboflavin by mouth daily, since studies by Dalgliesh(9) had pointed out the role of these vitamins in metabolism of tryptophan. None of the patients taking tryptophan experienced any severe side

TABLE I. Xanthurenic Acid Excretion in Control Patients.

Sex	Age	Before trypt. Vol	Creat.	XA	After trypt. 1 Vol	Creat.	XA	After trypt. 2 Vol	Creat.	XA	After trypt. + B_6 Vol	Creat.	XA
♂	49	3100	1.2	3.9	2000	1.0	15.8				2600	1.1	13.6
♀	69	1280	1.0	3.1	1680	1.1	5.8	1200	1.0	7.2	1720	1.1	7.5
♂	67	750	.9	5.9	1490	1.0	14.9				1110	1.2	7.0
♂	74	530	.9	5.6	1100	1.8	15.7				575	.8	5.2
♂	19	900	.6	3.2	1740	1.7	9.9	1240	.9	11.9	1300	1.2	8.4
♂	62	1220	1.1	7.8	1440	.9	7.1	840	.6	7.1	1880	1.0	10.4
♂	78	2660	1.4	7.8	1160	1.2	22.2				2610	2.5	18.0
♂	42	2240	.7	1.0	880	.5	9.2	1470	1.2	5.3	420	.5	1.0
♂	46	550	1.1	2.1	1670	1.0	10.2	2370	1.3	17.0	2200	1.6	7.0
♀	30	700	.9	8.8	1560	.9	14.2				1310	1.2	12.3
♀	80	1340	.6	2.8	1140	1.0	11.9				840	.8	10.5
♂	25	1185	1.8	2.6	1745	1.4	6.1				680	1.1	1.2
♂	72	1000	1.2	8.0	800	1.2	17.8	980	1.5	18.8	1820	1.4	16.7
♂	44	1090	1.6	7.7	2550	1.5	20.1				1800	1.5	11.5

TABLE II. Xanthurenic Acid Excretion in Heart Failure Patients.

Sex	Age	Before trypt. Vol	Creat.	XA	After trypt. 1 Vol	Creat.	XA	After trypt. 2 Vol	Creat.	XA	After trypt. + B_6 Vol	Creat.	XA
♀	58	530	.6	3.8	840	.8	52.4	760	.7	74.7	700	.5	4.6
♀	75	750	.8	5.0	1000	.7	55.8	770	.5	101.0	900	.8	14.6
♂	75	435	.5	1.1	800	.6	12.0	1125	.5	40.9	1860	1.0	13.0
♂	42	1970	1.0	1.0	2670	.9	14.9	920	.6	22.2	860	.7	6.4
♀	50	430	.5	2.2	500	.5	43.6				800	.8	14.9
♂	40	1180	1.3	4.0	1100	1.4	77.9				920	1.1	8.8
♂	72	1940	1.1	9.6	2375	1.4	8.3	500	.9	31.8	510	1.2	4.2
♂	48	2370	2.1	3.3	655	1.4	24.9	450	.9	26.5	505	.8	2.9
♂	62	1220	.7	3.4	1030	1.1	8.2				780	.7	7.0
♂	68	1600	.8	4.5	1780	.9	69.0	1380	.7	42.3	2530	1.4	20.9
♂	63	2850	1.9	3.1	2590	1.5	38.6				1920	1.2	12.2
♀	72	395	.9	3.0	430	.5	37.8	795	.3	38.7	700	.5	2.6
♂	70	1838	1.1	3.2	1420	1.0	14.1	1320	.7	32.7	1370	.7	18.0
♂	59	540	1.0	7.3	510	.6	40.8				1585	.7	14.1

reactions, although 2 patients were omitted from the study because of persistent nausea and vomiting on each occasion they were given the material, no matter how it was disguised. No change in blood urea nitrogen, or liver function, was noted in any of the patients during the study. Serum glutamic-oxaloacetic transaminase determinations were carried out by the method of Cabaud *et al.*(10) on each patient, since this enzyme is known to be involved in pyridoxine metabolism. The correlation between the S.G.O. transaminase levels and xanthurenic acid excretion will be published separately.

Results. Tables I and II present results of xanthurenic acid excretion, creatinine, and volume of 24 hr urine collections on all subjects. Fourteen heart failure patients and 14 control patients were selected for analysis who fulfilled the following criteria: 1. There was a pre-tryptophan, a post-tryptophan, and a post-tryptophan and pyridoxine value. 2. The level of creatinine in all urine collections was 0.4 g per day or higher. Collections with levels of 0.3 g or less were considered as fair evidence of poor collection of urine and were discarded. Means and standard deviations for these subjects are shown in Fig. 1.

The heart failure group and control group

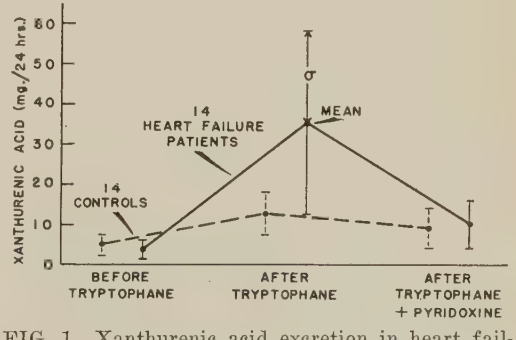

FIG. 1. Xanthurenic acid excretion in heart failure and control patients.

were quite comparable with respect to both mean and standard deviation in pre-tryptophan level of xanthurenic acid excretion with means of 3.9 and 5.0 mg/24 hr respectively. Following tryptophan load, each showed a highly significant mean increase over the pre-tryptophan level (probability of "t" less than 0.001 in each case). The mean increase for the heart failure group was, however, significantly greater than that for the control group (probability of "t" less than 0.01) so that the former reached a significantly higher mean level of excretion (35.6 *vs.* only 12.9 mg/24 hr). In addition, the variance for the heart failure group was considerably and significantly greater than that of the control group (standard deviation of 22.8 and 5.2 mg/24 hr respectively). This greater variance in the heart failure group was to be expected because these patients differed greatly in severity of condition while the control group was more homogeneous in the sense that none suffered from heart failure. The highest level attained by a control patient was 22.2 mg/24 hr while two-thirds of the heart failure patients exceeded this value.

When a tryptophan load was given after pyridoxine, the level was, for each group, significantly lower than with tryptophan load alone but significantly higher than in the pre-tryptophan period. In 20 of the 28 subjects, this pattern (lowest in the pre-tryptophan, highest in the post-tryptophan and intermediate in the post-tryptophan plus pyridoxine period) was followed exactly. The heart failure group, which had attained a higher level than the control group in the post-tryptophan period, showed a greater decline from post-tryptophan to post-tryptophan plus pyridoxine so that the 2 groups were at approximately the same level after pyridoxine (10.3 mg/24 hr for the heart failure group and 9.3 mg/24 hr for the control group). They also showed comparable variances after pyridoxine (standard deviation of 5.9 and 5.0 respectively). While the level after tryptophan plus pyridoxine was higher than that in the pre-tryptophan period, the difference was significant only in a statistical sense; however, it is apparent that pyridoxine decreased xanthurenic acid excretion levels comparable to those of the control pyridoxine treated group.

There were 16 subjects (8 heart failure and 8 control) to whom a second tryptophan load had been given. In these patients, the difference between results of the first tryptophan and second tryptophan loads were analyzed. The heart failure group showed an additional mean rise of 11.8 mg/24 hr with a standard deviation of 21.0, the control group showed an additional mean rise of 1.7 mg/24 hr with a standard deviation of 3.1. This indicates that the tryptophan load caused a true biochemical abnormality and not an artefact, and that in fact, abnormality became more severe after a second load test. The ability of pyridoxine to restore the body's response to tryptophan to normal in both controls and heart failure must be considered a specific effect.

Discussion. The statistically significant high levels of xanthurenic acid excretion in patients with congestive heart failure, tend to indicate that the nutritional status of the patients with respect to Vit. B_6 was subnormal. The biochemical response to administration of pyridoxine with improvement in derangement of tryptophan metabolism would further strengthen this conclusion. The only reference in the literature in regard to tryptophan metabolism in cardiac patients is that by Wachstein and Lobel(11); they found in 4 patients with cardiac decompensation, 24 hour urinary excretion of xanthurenic acid after a tryptophan load varying between 3 and 19 mg (3 mg, 10 mg, 14 mg and 19 mg respectively). These figures parallel some of our non-cardiac controls. It is to be regretted that no data are given as to the clinical status of the patients or as to whether compensation was restored by the conventional cardiac therapy. Furthermore, the patient in whom xanthurenic acid excretion after the tryptophan load was 19 mg responded to 100 mg of pyridoxine with zero xanthurenic acid excretion. We studied tryptophan metabolism in 9 patients after compensation was restored with bed rest, salt free diet, diuretics and digitalis. After restoration of compensation, xanthurenic acid excretion was within the limits of our controls (Table III). The question arises as to the factors accounting for the ab-

TABLE III. Xanthurenic Acid Excretion in Compensated Heart Failure Patients.

Sex	Age	Before trypt. Vol	Creat.	XA	After trypt. Vol	Creat.	XA	After trypt. + B_6 Vol	Creat.	XA
♂	46	550	1.1	2.1	1670	1.0	10.2	2200	1.6	7.0
♀	70	838	1.1	3.2	800	.75	17.4	600	.7	10.5
♂	74	503	.9	5.6	1100	1.8	15.7	575	.7	5.2
♀	70			8.9	520	.8	8.3	1620	1.1	1.8
♂	60	1200	.5	1.2	1260	.9	15.2	1240	1.0	14.9
♀	60	2480	1.4	3.9	970	1.0	18.2	900	1.0	10.3
♀	65	2380	1.4	9.7	2045	1.1	19.0	1800	1.1	13.5
♀	58	1985	1.5	7.9	2080	1.6	18.1	2100	1.6	18.5
♀	68	2930	1.2	4.3	3070	2.1	11.3	2425	1.2	7.7

normal tryptophan metabolism in congestive heart failure. It is quite likely that the basic difficulties in absorption and utilization of food factors present in heart failure may play an important role. The anorexia with inadequate food intake, anoxia of the intestinal tract due to passive congestion with resultant interference of absorption of pyridoxine supplied by the inadequate diet, may be a further reason for the abnormal tryptophan metabolism. That a similar situation exists with regard to thiamine in heart failure was pointed out(12). On the other hand, the abnormal tryptophan metabolism may indicate only a relative deficiency of pyridoxine: rate of synthesis of pyridoxine-containing coenzymes in the body may be less than rate of utilization.

The patients considered in these studies did not show gross clinical manifestations of pyridoxine deficiency. It is possible that pyridoxine deficit may occur in cardiac muscle not unlike that of thiamine, whereas peripheral organs may be free of that deficiency.

The limited studies available on pyridoxine metabolism in heart failure are indicative of the need for much more extensive investigation particularly at the blood and tissue level both from a clinical and laboratory point of view. Likewise, an evaluation of the effect of daily pyridoxine supplementation of the conventional therapy for congestive failure is desirable.

Summary. 1. Fourteen patients with congestive heart failure and 14 control patients without heart failure were studied with the tryptophan load test. 2. Urinary xanthurenic acid excretion following tryptophan load was significantly greater in the cardiac group than in controls. Mean XA excretion of the group with congestive failure was 35.6 mg/24 hours with standard deviation of 22.8. Mean excretion of the control group was 12.9 mg/24 hours with standard deviation of 5.2 mg/24 hours. 3. The heart failure group, which attained a higher level of XA excretion than the control group in the post tryptophan period, showed a greater decline in XA excretion after pyridoxine than the controls. 4. Since pyridoxine is required for orderly catabolism of tryptophan and pyridoxine corrects this metabolic aberration of tryptophan, it is not unreasonable to state that in congestive heart failure availability of Vit. B_6 is limited.

We acknowledge our appreciation to Dr. H. Menduke for statistical analysis of the data and to Elsie M. Faust for technical assistance.

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Gastric Ulceration in Rats with Experimentally-Induced Polycythemia. (25038)

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Increased incidence of peptic ulcer in patients with polycythemia rubra vera has never been explained satisfactorily. First reported by Friedman(1) it has been confirmed by many clinical investigators. These include Wilbur and Ochsner(2) and Lawrence(3) who observed peptic ulcer in 8 and 13%, respectively, of their large series of patients with polycythemia rubra vera, as compared with 2 to 3% in control groups of patients with other primary disorders. Boyd(4) suggested that the mechanism of ulcer formation is related to the well-documented(5) tendency to thrombosis also present in patients with polycythemia rubra vera. He postulates that local thrombosis within the vasculature supplying gastric and duodenal mucosa, leads to tissue necrosis and subsequent digestion by the gastric juice. Microscopic studies of ulcers in these patients, however, have failed to demonstrate consistent evidence of the postulated local thromboses(4). As our first step an attempt has been made to determine whether a similar relationship between polycythemia and ulceration could be demonstrated in the experimental animal.

Methods. Male rats of Sprague-Dawley and Holtzman strains, weighing 250 to 300 g, were utilized. Included were adrenalectomized, hypophysectomized, and sham-adrenalectomized and sham-hypophysectomized animals in addition to normal rats. They were rendered polycythemic by intravenous administration of washed and packed homologous rat erythrocytes. The erythrocytes were administered in 3 doses, at 2-day intervals, to a total dosage of 8% of body weight. In 2 control groups, normal rat plasma and isotonic saline, in equal amounts and administered in identical fashion, were substituted for the packed erythrocytes. By the sixth day, hematocrit levels greater than 75% were observed in all animals that received erythrocytes, whereas hematocrit levels of other groups showed no consistent change from preinjection values. All animals were autopsied 3 days following final infusion.

Results. Apart from generalized hyperemia, abnormalities in polycythemic animals were confined to the stomach (Table I). The stomachs of 26 of the 29 animals, including all 14 adrenalectomized, hypophysectomized, and sham-operated animals, showed ulceration that resembled human peptic ulcers both

TABLE I. Gastric Findings at Autopsy.

Type of infusion	Type of rats used	No. of animals			Normal animals
		Total	Ulcer	Gastritis	
Packed erythrocytes	Normal	15	12	3	0
Idem	Adrenalectomized	5	5	0	0
"	Hypophysectomized	5	5	0	0
"	Sham-adrenalectomized	2	2	0	0
"	Sham-hypophysectomized	2	2	0	0
	Totals	29	26 (90%)	3 (10%)	0 (0%)
Normal rat plasma	Normal	6	0	0	6
Isotonic saline	"	6	0	0	6
No infusion	"	10	0	0	10
Idem	Adrenalectomized	2	0	0	2
"	Hypophysectomized	2	0	0	2
"	Sham-adrenalectomized	2	0	0	2
"	Sham-hypophysectomized	2	0	0	2
	Totals	30	0	0	30 (100%)

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FIG. 1. Microscopic appearance of section of ulcer observed in polycythemic rat. Hematoxylin, eosin, and azure II stain (× 34).

FIG. 2. A more chronic-appearing lesion demonstrating granulation tissue and fibroblastic proliferation. Hematoxylin, eosin, and azure II stain (× 42).

grossly and microscopically. The ulcers were located in the body and antral regions of stomach, and were single in 22 and multiple in 4 animals. They were round or oval and ranged from a mm to over a cm in diameter. The craters were sharply circumscribed, with dark bases. Stomachs of the 3 remaining rats of this group, although not exhibiting discrete ulceration, were nevertheless abnormal, demonstrating multiple small areas of superficial hemorrhagic erosion along the tops of the rugal ridges. No lesions were present in any animal of either of control groups.

Microscopic study of ulcerations revealed typical fibrino-necrotic crater base demarcated sharply from surrounding normal-appearing mucosa. The more acute-appearing lesions were characterized by intact muscularis mucosae (Fig. 1). The associated leucocytic infiltration extended in some instances entirely through the serosa to peritoneal surface, and was accompanied by moderate to marked intercellular edema. The more chronic-appearing ulcers extended through the muscularis mucosae and demonstrated crater bases composed of granulation tissue with underlying fibroblastic proliferation (Fig. 2). This type of ulcer showed a tendency to bleed massively into the bowel, resulting in hematocrit reductions to 45% overnight.

Thrombosed blood vessels were present in only 1 specimen. This showed a small vein and a small artery thrombosed, both located at base of crater, within the necrotic layer. In this location, the etiologic significance is questionable, for, as observed by Anderson(6) in his description of human peptic ulcer, these may have occurred secondary to ulceration. Stomachs of the 27 remaining animals of the polycythemic group showed no evidence of thrombosis in regions studied microscopically.

Discussion. These experiments demonstrate, therefore, that gastric ulceration in the rat can be produced by transfusion-induced polycythemia. Absence of demonstrable vascular thrombosis in all but one of the lesions suggests that additional factors may be involved in their pathogenesis.

Gastric ulceration was reported by Highman and Altland(7) in a small percentage (5 of 79) of rats exposed to simulated high-altitude and thereby rendered secondarily polycythemic. However, it is not clear whether the ulceration observed was related to the non-specific stress induced by hypoxia or to the polycythemia. Indeed, the stress of hypoxia may have been the important factor since Reynolds and Phillips(8) reported gastric ulceration in rats subjected to simulated

high-altitude which did not become frankly polycythemic.

Since adrenalectomy and hypophysectomy did not abolish the ulcerogenic effect of transfusion observed in our study, it seems unlikely that a non-specific stress reaction, usually thought to be mediated through the pituitary-adrenal axis(9,10), accounts for the results we observed.

It is possible that the relative circulatory stasis present in the distended vasculature of the polycythemic animals, similar to that discussed by Brooks(11) in studies on circulatory adjustments in human polycythemia rubra vera, may diminish tissue resistance sufficiently to allow ulceration even in the absence of frank thrombosis and mucosal infarction. Alternatively, by allowing CO_2 to accumulate in the gastric mucosa as a product of cellular metabolism, stasis may predispose to ulceration by increasing hydrochloric acid secretion via the carbonic anhydrase-dependent equilibrium: $CO_2 + H_2O \leftrightarrows H^+ + HCO_3^-$, which may be the basic mechanism of gastric acid secretion as recently demonstrated and emphasized by Davies(12) and Janowitz(13). The attractiveness of this latter speculation is enhanced by the observation that ulcers in patients with polycythemia rubra vera are largely (90%) duodenal(2,3), a location that has been most often associated with gastric hypersecretion of acid(14).

These findings suggest an interesting parallel between our experimental system and the patient with polycythemia rubra vera and its associated increased incidence of peptic ulcer. Additional studies are in progress to determine whether gastric hypersecretion of acid occurs secondary to the induced polycythemia, as we have postulated, or whether, in its absence, vascular factors alone may initiate ulcer formation.

Summary. Induction of polycythemia by repeated intravenous injections of homologous erythrocytes produced gastric ulceration in 26 of 29 rats, including normal, adrenalectomized, hypophysectomized, and sham-operated animals. An experimental system has therefore been developed for the study of etiologic factors which may parallel those leading to development of peptic ulcers in patients with polycythemia rubra vera.

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Effect of Norepinephrine and Epinephrine on Nonesterified Fatty Acid Concentration in Plasma. (25039)

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It has been suggested that the sympathetic nervous system has a role in regulation of fat transport(1). Fatty acids are transported in plasma, partially in nonesterified form(2,3). Their source in a fasting animal appears to be adipose tissue(4-6). Injection of epinephrine increases plasma nonesterified fatty acid (NEFA) concentration(2,3,7) by enhancing

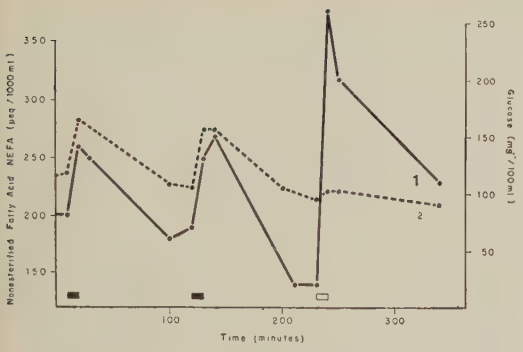

FIG. 1. Effect of epinephrine and norepinephrine on concentrations of plasma NEFA (Curve 1) and glucose (Curve 2). ■, infusion period of epinephrine; □, infusion period of norepinephrine.

hydrolysis of triglycerides to fatty acids in adipose tissue(8). Recently, it has been shown that addition of norepinephrine, a chemical mediator of sympathetic nerve endings, to flasks containing epididymal adipose tissue enhances hydrolysis of esterified fatty acids(9). The present report demonstrates that norepinephrine, as well as epinephrine, when infused into dogs, raises plasma NEFA concentration.

Method. Catheters were inserted in each femoral vein of 4 mongrel dogs, weighing 11 to 13 kg and fasted 24 hours and anesthetized with sodium pentobarbital. Solutions of epinephrine or norepinephrine bitartrate in 0.85% sodium chloride, adjusted to pH 5 with 0.1 N HCl, were made just before starting the infusion. A solution containing 10 μg base/ml was infused through one of the catheters at constant rate of 8.4 μg/min. for 10 minutes. Blood samples were withdrawn from opposite femoral vein 10 minutes before infusion was begun, at beginning, and 10, 20, and 90 minutes after infusion was started. Five ml of blood was immediately placed in a tube containing 0.5 ml of 3.8% sodium citrate, and the sample mixed and chilled in an ice bath. Plasma obtained by centrifugation was analyzed for NEFA(10) and glucose(11). The method of NEFA analysis excluded water-soluble organic acids.

Results. The data from one typical experiment are shown in Fig. 1. Infusion of epinephrine for 10 minutes increased initial plasma NEFA concentration by about 35%; followed by a slight decline 10 minutes after infusion was stopped. Repeating the infusion of epinephrine, in the same dog, gave essentially the same result. However, infusion of norepinephrine at the same concentration and rate increased the initial NEFA concentration almost 2-fold at the end of 10 minute infusion period; 10 minutes after infusion was stopped the concentration decreased slightly, and within 90 minutes it decreased considerably but remained higher than the initial NEFA concentration.

During infusion of epinephrine changes in the glucose concentration of plasma paralleled changes in NEFA concentration. In contrast, norepinephrine produced little change in plasma glucose concentrations although large changes in NEFA concentrations were noted.

Similar results were obtained in 3 other experiments. Reversal of the order of epinephrine and morepinephrine administration had no effect.

Discussion. White and Engel(9) have shown that both norepinephrine and epinephrine at same concentration stimulate hydrolysis of neutral fats to fatty acids to the same extent in adipose tissue incubated *in vitro*. Similar observations have been made by us. Therefore, it would be expected that infusion of epinephrine and norepinephrine into animals would have the same effect on plasma NEFA. However, in all 4 experiments, the increase in NEFA was greater when norepinephrine was infused. It is possible that the difference in response of plasma NEFA is due to faster destruction of epinephrine, or to an inhibitory effect of increased plasma glucose concentrations after infusion of epinephrine on release of NEFA from adipose tissue(5).

That infusion of norepinephrine and epinephrine produces an increase in plasma NEFA concentration suggests that these compounds may function as circulating hormones, acting directly on adipose tissue to regulate fatty acid mobilization. This suggestion is substantiated by the observation that adipose tissue from adrenalectomized rats, incubated *in vitro,* undergoes less hydrolysis of esterified fatty acids. The fact that norepinephrine is more effective than epinephrine is consistent with the hypothesis that sympathetic nervous

system is involved in regulation of fat transport due to release of norepinephrine at nerve endings in adipose tissue.

Summary. Concentrations of nonesterified fatty acids and of glucose in plasma were determined before and after infusion of epinephrine and norepinephrine into dogs. At the same concentration and rate of infusion, norepinephrine produced a greater increase in nonesterified fatty acids.

ADDENDUM: Since completion of this manuscript, we found that Laurell, S., Christensson, B., *Acta Physiol. Scand.,* 1958, v44, 248 reported increased plasma NEFA in human subjects following norepinephrine injection.

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Periarteritis in Rats Given Single Injection of 4′-Fluoro-10-methyl-1,2-benzanthracene.* (25040)

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Periarteritis nodosa has been recognized in patients for almost a century(1), and an etiology of hypersensitivity has been invoked (2-4). Thus, the disease sometimes appears to follow administration of foreign sera(4,5), and similar lesions can be induced in experimental animals by foreign proteins(6-11). A number of clinical cases of periarteritis nodosa have been ascribed to hypersensitivity reaction to various drugs (i.e., sulfonamides(12) and iodides(13)). Similar vascular lesions have been induced in experimental animals by a few compounds of low molecular weight. Periarteritis was observed in rats and monkeys stressed by unilateral nephrectomy and high salt diet and then given 9-fluoro- or 9-chloro-2-methylcortisol(14-16). Anemic infarction of lungs was found in rats treated with certain halogenated hydrocarbons such as hexachlorotetrafluorobutane(17). In a study on carcinogenicity of various fluorinated derivatives of 10-methyl-1,2-benzanthracene (10-Me-BA)† all rats injected subcutaneously with 2.14 mg of 4′-fluoro-10-Me-BA died within 8 weeks. A marked degree of vascular changes as found in lungs and kidneys, and pulmonary lesions appeared to cause death. Development of these lesions was followed in a second series of rats, and data for the latter experiment form the subject of this report.

Materials and Methods. Male adult albino rats of Holtzman stock‡ with average initial weight of 250 g were maintained in screen-bottom cages in groups of 2 to 4 and were fed Purina Laboratory Chow *ad lib.* Each rat received a single subcutaneous injection of 0.2 ml of tricaprylin (previously dissolved in petroleum ether and extracted with alkali to remove traces of free acid) or 0.2 ml of tri-

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† Carcinogenicity studies are being made in collaboration with Dr. Melvin Newman of Ohio State Univ., to whom we are indebted for hydrocarbons used.

‡ Holtzman Rat Co., Madison, Wis.

TABLE I. Number of Rats with Periarteritis at Various Times.

Weeks after inj.	Hydrocarbon inj.* None	10-Me-BA, 2 mg	4′-Fluoro-10-Me-BA 2.14 mg	4′-Fluoro-10-Me-BA 1.07 mg
0	0/3†			
1		0/2	0/3	
2	0/2	"	1/4	0/2
3		"	0/3	
4	"	"	4/7	"
5		"	4/5	
6	"	"	2/2	1/2
7		"	"	
8	0/3	0/4		2/4
Total	0/12	0/18	13/26	3/10

* Tricaprylin served as the solvent for subcut. inj. of the hydrocarbons and was also administered to control rats.

† Numerator denotes No. of rats with periarteritis; denominator, No. of rats autopsied.

caprylin containing 2 mg of 10-Me-BA, 2.14 mg of 4′-fluoro-10-Me-BA (equimolar to the 10-Me-BA), or 1.07 mg of 4′-fluoro-10-Me-BA. At weekly intervals for 8 weeks the rats were weighed and representative animals (2-4/group) were killed (Table I). Autopsies were performed and pieces of major tissues, except brain, were fixed in 10% formalin. The sections were cut at 6 μ and stained with hematoxylin-eosin, periodic-acid-Schiff (PAS) reagent(18), and Kingsley stain (19). On microscopic examination the vascular lesions in the organs were graded from 1+ to 4+. Grade 1+ designates very mild lesions with little or no perivascular infiltration. Grade 4+ designates pronounced periarteritis which involves all 3 layers of the vessels and is associated with necrosis and thickening of vessel walls. Grades 2+ and 3+ are of intermediate severity. Only lesions of 2+ or greater severity were considered of diagnostic significance.

Results. The general health of control rats and those injected with 10-Me-BA was good; these rats gained an average of 130 g during the 8-week experimental period. On the other hand, rats injected with 2.14 mg of 4′-fluoro-10-Me-BA gained 26 g during the first 2 weeks and thereafter lost weight slowly. From the fifth to seventh weeks surviving rats averaged 5-10 g below their starting weights. Weights of rats injected with the lower dose of 4′-fluoro derivative were similar to those of control rats until the fifth week; thereafter, their weights remained essentially constant. Rats killed for autopsy 4 to 7 weeks after injection of 2.14 mg of 4′-fluoro-10-Me-BA were generally in poor health; some were cyanotic and appeared to be near death.

The major pathological lesion was periarteritis (Table I) which involved particularly the vessels of lungs and kidneys, but occasionally also, to a lesser extent, the periadrenal and pancreatic arterioles. In the lungs lesions were most pronounced in arteries whereas in kidneys arterioles and capillaries were involved. The lesions were first observed in lungs of a rat killed 2 weeks after injection of higher dose of 4′-fluoro-10-Me-BA, but they were sporadic and poorly developed. By the fourth week 4 of 7 animals examined had 3-4+ lesions in pulmonary and/or renal vessels, and all but one of the animals killed thereafter had advanced lesions. Of the animals injected with 1.07 mg of 4′-fluoro-10-Me-BA 3 of 6 rats examined at 6-8 weeks had these lesions. No vascular lesions were observed in any rats which received injections of tricaprylin alone or tricaprylin containing 2 mg of 10-Me-BA.

Lungs. In the larger arteries there was a marked thickening of all 3 layers (Fig. 1). The intima was characterized by endothelial proliferation which tended to occlude the lumen. The media was edematous and infiltrated by numerous inflammatory cells, of which the majority were neutrophilic, polymorphonuclear leukocytes and fibroblasts. Eosinophilic leukocytes were very scarce, even in sections stained with the Kingsley preparation. Nuclear debris and pyknotic and karyorrhectic nuclei were frequently scattered among the leukocytes. While the internal elastic membrane was fairly distinct, there was no sharp distinction between media and adventitia. Similarly, the outer layer of adventitia blended in with the inflammatory infiltration of adjacent lung tissue. Necrosis was rare and when it occurred it involved only a segment of the arterial wall.

The PAS reaction stained the intima more strongly than other layers. It outlined cell membranes clearly, but did not indicate an excessive deposition of polysaccharides.

Kidneys. Lesions in kidneys selectively involved arterioles and extended into the glom-

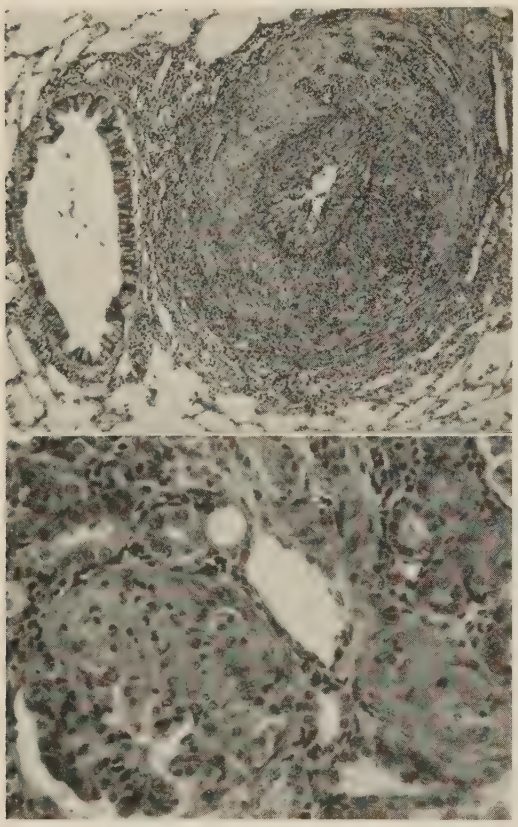

FIG. 1 (top). Periarteritis of a pulmonary artery. Endothelial proliferation, as well as inflammatory infiltration and fibrosis of the media and adventitia are evident. Grade 3+ lesion. (100×, PAS stain.)

FIG. 2 (bottom). Hyaline necrosis of kidney arterioles and capillaries. Afferent arterioles at the right are almost occluded. Upper half of the glomerulus at left shows obliteration of the capillaries. Grade 3+ involvement. (100×, PAS stain.)

erular capillaries (Fig. 2). Arterioles, particularly the afferent arterioles, exhibited hyaline necrosis which tended to obstruct the lumen and extended into the glomerular capillaries, where the degree of involvement varied from only a few loops to the entire glomerulus. In some cases glomeruli were swollen and adherent to outer layer of Bowman's capsule. These lesions are not specific for periarteritis since they have been described in malignant nephrosclerosis and glomerulonephritis as well(20). Fundamental similarities in the pathogenesis of glomerulonephritis and periarteritis have been suggested by Masugi and Sato(6), Rich(4) and others who have stressed their frequent concurrence. We believe that in this case association of glomerular changes with a more typical lesion in the lungs favors a diagnosis of periarteritis.

Renal lesions were most pronounced in rats killed in fourth and fifth weeks and were absent from those killed at 7 and 8 weeks. This observation suggests that the lesion may be reversible. In this connection it is of interest that Hawn and Janeway(21) observed regression of the vascular lesions induced in rabbits by administration of foreign proteins; regression of lesions was correlated with a decreased level of circulating antigen.

Discussion. The etiology of severe and fatal periarteritis which occurs in rats given a single injection of 4′-fluoro-10-Me-BA is not known. Similar lesions were not seen in rats given equimolar doses of the parent hydrocarbon 10-Me-BA, and 3-fluoro-10-Me-BA was also non-toxic under same conditions.§ The stability of C-F bonds of this type and the lack of reports of a similar lesion in fluoride-treated rats suggests that the fluorine atom itself is not involved. Hypersensitivity has been shown to be an etiological factor in periarteritis nodosa, and the possibility that an immunological response may occur in rats given 4′-fluoro-10-Me-BA should also be considered. Williams(22) reported that a human volunteer treated topically at one site with 9,10-dimethyl-1,2-benzanthracene and then, 1 and 2 months later, treated similarly at other sites showed, subsequent to second and third applications, a papulo-vesicular response both at test site and in areas previously painted with the hydrocarbon. No response occurred after first application. Various polycyclic aromatic hydrocarbons(23-26) have been shown to combine with certain tissue proteins of the mouse *in vivo*. If 4′-fluoro-10-Me-BA likewise combines with one or more proteins *in vivo* and should yield an antigenically foreign protein, autoimmunization would result. Such an autoimmunization against altered tissue proteins has been suggested by Green(27) as the basic factor in genesis of cancer by polycyclic hydrocarbons and other chemicals.

Summary. 1. A single subcutaneous injection of 2.14 mg of 4′-fluoro-10-methyl-1,2-benzanthracene in albino rats produced a se-

§ Miller, J. A., Miller, E. C., unpublished data.

vere and fatal periarteritis in 4 to 8 weeks. Similar lesions were seen after injection of one-half as much of this hydrocarbon, but they developed more slowly. Equivalent levels of 10-methyl-1,2-benzanthracene or the solvent alone were ineffective. 2. The most severe lesions were observed in pulmonary arteries; this finding is in accord with the cyanotic conditions of some rats. Hyaline necrotic lesions were observed in renal arterioles and glomerular capillaries. 3. A possible immunological basis for these lesions is discussed.

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Comparative Anti-Inflammatory Efficacy of Topically Applied Steroids on Human Skin. (25041)

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Hundreds of hydrocortisone analogs have been synthesized in the search for more ideal anti-inflammatory agents. It is desirable to evaluate these newer compounds in animals and humans as quickly as possible. This paper describes results obtained using a modification of an unpublished assay method recommended by Brunner and Finkelstein (Research Laboratories, The Toni Co., Chicago). The original method will be reported by these authors.

Method. Immediately prior to testing, 7 accurately weighed steroid samples are separately dissolved in 15 ml tetrahydrofurfuryl alcohol (THFA).* This irritant alcohol serves both as solvent for test steroids and as agent for producing experimental erythema. Potencies of the steroids are determined from their ability, when applied simultaneously with the irritant, to inhibit THFA-induced contact dermatitis. THFA alone is used as control. Twenty-four white male volunteers at least 21 years of age are used for each test. One by 3″ Elastoplast† bandages are used to

* Quaker Oats Co., Chicago, Ill.

† Duke Labs., South Norwalk, Conn.

TABLE I. Comparison of Anti-Inflammatory Potency of Steroids Determined by Experimental Method with Potencies Determined by Clinical Trial or Wide Dermatological Use.

Test steroid	Estimated potency (× 1% hydrocortisone) with S.E.	Clinical potency (× 1% hydrocortisone)
Cholesterol	0	0
Cortisone	.04*	Not detectable†
Hydrocortisone	1.0	1†
Prednisolone	1.8 ± .5	2†
6α-methylprednisolone	4.7 ± 1.3	4‡
9α-fluorohydrocortisone	9*	10†
6α-methyl-9α-fluoro-21-deoxyprednisolone (fluorometholone‖)	42.0 ±11.0	40§

* Not tested sufficiently for reliable estimation of stand. error.
† See ref. 1.
‡ " " 2.
§ Unpublished data from Dept. of Clinical Investigation, Upjohn Co.
‖ Oxylone: Registered trademark, Upjohn Co.

apply 0.25 ml of each solution. These treatments are assigned to subjects and to positions on forearms so that effects associated with subjects and positions can be eliminated from treatment comparisons. The actual design constitutes a set of three 8 x 8 Latin Squares. With this design each steroid sample and the control is tested at one site on each volunteer. For a quantitative assay, 3 or 4 concentrations of the unknown are compared with 3 concentrations of hydrocortisone, the reference steroid. Test solutions are in contact with the skin from late afternoon until next a.m. Assay sites are scored visually according to degrees of developed erythema using an erythema scale of zero to 4. A zero score indicates no erythema or a completely inhibited reaction. Scores of 1, 2, and 3 represent minimal, mild, and moderate degrees of erythema. A score of 4 denotes a pronounced erythema or no inhibition of the THFA induced reaction. With certain exceptions, one should refrain from retesting the same individuals to avoid more severe and aberrant responses. The mean erythema score for each steroid concentration subtracted from mean control value yields a numerical improvement value (response) used in evaluating relative potencies of test steroids.

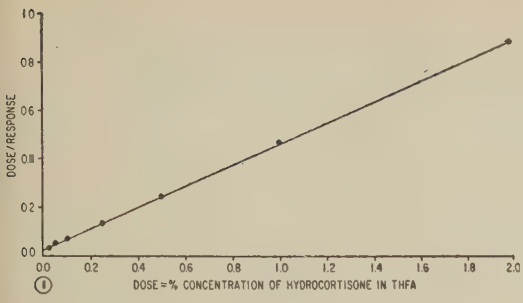
Results. During the test an uninhibited erythema develops at control sites treated with THFA alone. At sites previously occupied by the steroids dissolved in THFA, the erythema is checked completely or held at various degrees of abeyance depending upon potency and concentrations of the steroids being studied.

Several steroids have been tested sufficiently in multiple-dose assays to permit statistical analysis and more precise quantitation of potencies (Table I). This study reveals the very excellent agreement between potencies of steroids as estimated in this assay and potencies determined in topical treatment of steroid responsive dermatoses. These clinical values are based on what may be considered the average effective topical steroid concentrations. Estimated potency values were determined by quantitative technic described below.

Intensive study of assay data indicated that the theory of Stetten(3) concerning relationship of hormone dosage to physiological response applied to steroids of Table I. This finding has permitted comparison of steroids with respect to: (a) capacity of target organ to bind hormone, (b) affinity of hormone for binding sites, and has also determined, in part, the nature of statistical analysis and method of estimating potency.

According to Stetten's(3) theory, dosage divided by response should be a linear function of dosage for a given steroid and target organ. Thus: (1) $D/R = A + B \cdot D$, where D = dose, R = response, A is the intercept on the D/R axis, and B is the slope of line. The value of $1/B$ is a relative measure of capacity (total number of sites for physiological responsive attachment of hormone to target organ). The value of B/A is a relative measure of affinity of hormone for binding sites.

Evidence for applicability of Stetten's(3) approach is shown in Fig. 1 where ordinates, D/R, were calculated in one assay by taking R as mean improvement over control at given concentration of hydrocortisone for 24 volunteers. Such plots of D/R against D for the



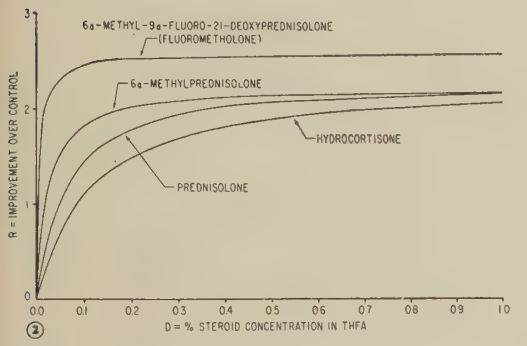


FIG. 1. Relationship of dose/response to dose for hydrocortisone in THFA.

FIG. 2. Estimated response/dose curves for four steroids according to theory of Stetten; $R = D/(A + B \cdot D)$.

anti-inflammatory steroids usually give linearity and also homogeneous variation in dose/response.

Estimates of A and B for each steroid have been obtained by the method of least squares applied to the plot of D/R against D. Table II summarizes these estimates for 4 of the steroids studied.

Within the framework of Stetten's theory, certain conclusions may be obtained from Table II. Values of B are statistically fairly similar for hydrocortisone, prednisolone, and 6α-methylprednisolone; this indicates that the target organ exhibits equal capacity to bind these steroids. However, the B value for fluorometholone is statistically significantly lower (beyond the 5% level) than those obtained for other steroids. Hence, the target organ has greater capacity to bind fluorometholone than it has to bind the other 3 steroids. The capacity to bind fluorometholone relative to hydrocortisone is .431/.385 or 1.12 ± .03.

This 12% increase in capacity for fluorometholone indicates that a greater anti-inflammatory response should be obtained with maximum effective concentration of fluorometholone than with maximum effective concentration of hydrocortisone. The increased capacity has great practical significance, since in treatment of severe or resistant inflammatory dermatoses, fluorometholone should be capable of producing a greater anti-inflammatory response than that possible with even very high concentrations of hydrocortisone. Of even greater significance is the fact that, while fluorometholone is 42 times as effective as hydrocortisone topically, it is only about equal to hydrocortisone by oral or intravenous administration(4,5). This suggests that fluorometholone should be an ideal steroid for highly effective topical anti-inflammatory treatment without the danger of any deleterious hormonal side-effects due to systemic activity.

Among those compounds which have a common B value, the ratio of A values gives an estimate of relative steroid potency (Table III).

However, the relative potency of steroids with different capacities (different values of B) cannot be determined on the basis of the ratio of the A (intercept) values. Therefore, the potency of fluorometholone was calculated from equations in Table II for fluorometholone and hydrocortisone, or determined graphically from smooth graphical plots of R against D [from $R = D/(A + B \cdot D)$] for these 2 steroids (Fig. 2). From these plots it was readily determined that 0.024% fluorometholone gave the same response as 1% hy-

TABLE II. Estimates of Parameters in Stetten's Theory for 4 Steroids.

Steroid	D/R =	A	+ B·D	Stand. error of A	Stand. error of B
Hydrocortisone	D/R =	.0480	+ .431D	.0058	.005
Prednisolone	" =	.0271	+ .425D	.0074	.014
6α-methylprednisolone	" =	.0103	+ .443D	.0026	.014
Fluorometholone	" =	.00225	+ .385D	.0004	.009

TABLE III. Estimated Potency of Prednisolone and 6α-Methylprednisolone Relative to Hydrocortisone.

Steroid	Value of A	Potency relative to hydrocortisone	Stand. error of relative potency
Hydrocortisone	.0480	1	
Prednisolone	.0271	1.8	.5
6α-methylprednisolone	.0103	4.7	1.3

drocortisone. Hence, at the clinically useful 1% hydrocortisone level, fluorometholone was 1/.024 or 42 times as potent as hydrocortisone. Since these original observations were made, this high potency for fluorometholone has been demonstrated repeatedly in numerous additional assays where it has been compared both with hydrocortisone and with other steroids.

Summary and conclusions. 1. A reliable assay procedure has been presented for rapidly quantitating the anti-inflammatory potency of steroids on human skin. 2. Estimated steroid potency values can be used to predict reliably the proper steroid concentration to be studied in topical clinical trials. 3. Data have been adapted to the Stetten theory which concerns relationship of hormone dosage to physiological response. This permitted comparison of steroids with respect to capacity of target organ to bind hormone and affinity of hormone for binding sites. 4. The target organ has increased capacity to bind fluorometholone relative to hydrocortisone. This means that some optimum concentration of fluorometholone is capable of producing a greater anti-inflammatory response than that possible even with very high doses of hydrocortisone. 5. That fluorometholone is approximately 40 times as potent as hydrocortisone topically yet only about equal to hydrocortisone in systemic activity suggests that fluorometholone is a unique compound for topical anti-inflammatory therapy, since even if absorption from the skin occurred, the systemic effect would be negligible.

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Evolution of Herpes Simplex Cellular Lesions Observed *in vitro* by Phase Contrast Microcinematography. (25042)

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Since first attempts of *in vitro* cultivation of herpes simplex virus by Rivers *et al.*(1), Andrewes *et al.*(2), and others, many authors have described specific lesions produced by this virus in cells maintained *in vitro*. However, despite considerable amount of information accumulated so far, some points concerning phenomena occurring in herpes virus infected cells have remained controversial. Our purpose was to record by continuous observations with phase contrast microcinematography the herpes infectious process in living cells, and to compare these data with those obtained by routine histological techniques. We were thus able to follow step by step the interrelationships between different cell constituents and organelles during development of the herpetic cell lesion and, on an intercellular level, to study stage by stage the formation of giant cells characteristic of this infectious process.

Material and methods. Cultures of trypsinized rabbit kidney cells were prepared in

flattened tubes on 13 × 32 mm coverslips as previously described(3,4). The culture medium contained Morgan's 199 synthetic medium (57%), bovine amniotic fluid (30%), rabbit serum (10%) and bovine embryo extract (3%). The herpes simplex "5433" virus strain originating from a human case of acute stomatitis was adapted to, and has been carried in rabbit tissue cultures(3), since 1955. Since its first passages *in vitro* this strain has produced rapidly generalized lesions characterized by giant cell formation. For continuous phase contrast observation and microcinematography, the culture-bearing coverslips were mounted in special perfusion chambers(5) and maintained at body temperature on water-perfused heating stage(6). The Zeiss-Winkel phase contrast, long focal condensor, optical system was used. The microcinematography device has been described (7). The light source was a low voltage tungsten filament lamp with Wratten "60" green filter. Frequency of time lapse pictures varied from 1/sec. to 2/min., with 0.5 sec. exposure time. Cultures were light-protected between exposures by intermittent screening. Some microcinematographic records were analysed frame by frame and essential results computed by serial drawing. After observation in living state, cultures at different stages of infection were fixed in Bouin, Bensley or Pallade buffered osmic acid fixatives and stained with hematoxylin-eosin or ferrous hematoxylin. Feulgen and methyl green-pyronin stains were used after fixation with Carnoy solution. Virus titrations were performed by serial dilution method in cultures of rabbit kidney cells (3).

Results. As previously observed(3) trypsinized rabbit kidney tissue produced *in vitro* a mixed cell growth in which fusiform and polygonal cells were present along with typical epithelial sheets. The herpes virus "5433" strain when introduced at 10^5-10^6 ID50 concentrations in cultures containing 1 to 2 × 10^5 cells produced a generalized cytopathic effect with intense giant cell formation in less than 24 hours. No preferential attack of the virus upon epithelial or fibroblastic cells was observed.

The first detectable virus released from cultures, thoroughly washed after infection, was found in the medium at $10^{0.5}$ ID50/ml concentrations around 10th hour after infection. The virus titer increased to $10^{0.8}$-$10^{1.8}$ levels between 23rd and 28th hour. However massive accumulation of virus in the medium, attaining 10^6-10^7 ID50/ml levels occurred, starting from 48th-50th hour. A massive release of virus from cells followed with some delay, generalization of the nuclear lesions observed in cultures as early as 24 hours after infection.

The first discrete alterations in living infected cells could be observed in the nucleus. From 9-10th hour after infection nucleoli in many cells lost their optical homogeneity, becoming partially less opaque, lobulated and granulated. In this way the nucleoli disappeared gradually both by dissolution and disintegration (Fig. 4-6). Only isolated nucleolar debris stuck to the nuclear membrane and could be detected after fixation and staining as rarely dispersed pyronin-positive granules. Simultaneously and independently very dark, spherical granulations appeared throughout the nucleus. These formations corresponded in size and number to Feulgen and methyl green positive granulations seen in stained control preparations. The Brownian movement in the nucleoplasm became progressively more intense and granulations tended to accumulate close to the nuclear membrane, the outlines of which became thicker and less regular. All these changes were asynchronous in cells of the same culture, even when infected with highly concentrated virus suspensions.

Later a zone of optical densification could be observed in the central area of many nuclei, but in no case did this take on an aspect of a sharply delineated inclusion body (Fig. 1-3). Such inclusion bodies located in the nuclear center and well separated from the nuclear membrane by a clear halo could be identified however in many cells of the same cultures after fixation with Bouin or Bensley mixtures and hematoxylin-eosin staining. Yet no definite pictures of intranuclear inclusion bodies were seen when Pallade's buffered osmic acid mixture was substituted for the classical fixatives.

At initial stage of infection, during the 10-20th hour period, the cytoplasm of many cells

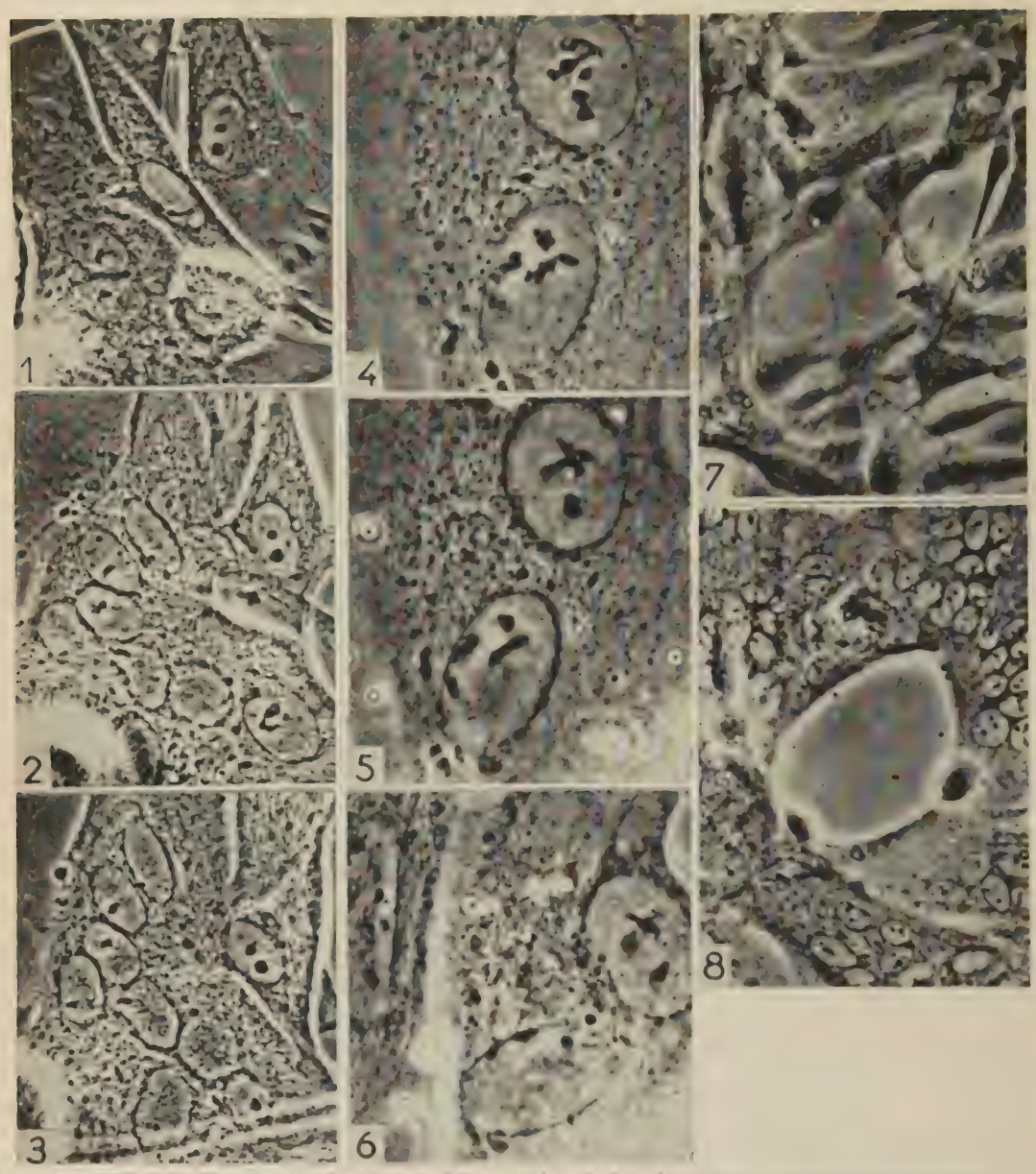

FIG. 1-3. Three successive stages of herpes virus lesions in a group of rabbit kidney cells, 8 hr 55 min., 10 hr and 10½ hr after infection; dissolution of intercellular limits, disappearance of nucleoli, optical densification of central nuclear area visible in last 2 pictures. (Phase contrast, magnif. 30 × 6.)

FIG. 4-6. Nuclei in herpes infected cells 10½ hr, 11 hr 50 min. and 15 hr 10 min. after infection; progressive dissolution and dispersion of nucleoli, increasing heterogeneity of nucleoplasm, thickening of nuclear membrane. (Phase contrast, magnif. 68 × 4.)

FIG. 7-8. Transformation of area of typical connective tissue cells into multinucleated plasmodes. Fig. 7—8½ hr following infection with herpes virus. 2 hr 45 min. elapsed between pictures 7 and 8. No nuclear division but only nuclear aggregation was seen in this area. (Phase contrast, magnif. 8 × 6.)

showing heavy intranuclear alterations kept its full activity as evidenced by persistence of normal filamentous mobile mitochondria, by presence of non-retracted cytoplasmic pseudopodia and by energetic activity of undulating membranes (Fig. 9-12). All this activity

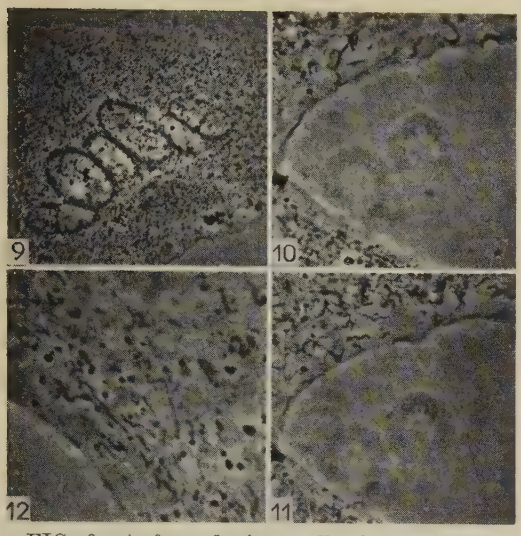

FIG. 9. A formed giant cell with typical herpetic nuclear lesions. (Magnif. 20 × 4.)

FIG. 10 and 11. Same cell as Fig. 9; ectoplasmic membranes in full activity, 10 min. elapsed between 10 and 11. (Magnif. 45 × 4.)

FIG. 12. Same cell as Fig. 9; cytoplasmic area with active mitochondria. (Magnif. 45 × 6.)

later ceased rather abruptly with a general cell retraction and arrest of movement in both cytoplasm and nucleus.

During the early 10-24 hour period, concurrently with the described intranuclear changes, very rapid and generalized giant cell formation, equally involving epithelial sheets and fibroblastic cell agglomerations was observed. The essential picture was that of progressive disappearance of cell boundaries and massive accumulation of nuclei in certain areas which were transformed into big plasmodes. This process occurred rather rapidly: thus formation of typical plasmodes containing 20 to 50 nuclei from apparently normal, isolated cells often occurred in no more than 2 to 3 hours (Fig. 7, 8).

We observed no nuclear duplication in areas of giant cell formation but were able to follow easily migration of nuclei through the cytoplasm. In certain cases this migration was so rapid that it could be checked by direct observation. Occasionally, following a retraction of cell sheets one notices a free flow of nuclei through the plasmode as if cytoplasm offered no more resistance than an astructural liquid of very low viscosity. Another indication of cytoplasm liquefaction during giant cell formation was the increasing intensity of random movement of intracytoplasmic granulations, especially in immediate vicinity of nuclei.

Some nuclei participating in giant cell formation had nucleoli in a state of dispersion whereas some nuclei appeared normal in this respect. Size of giant cells was variable, many containing 50 or more nuclei. No ordered accumulation of nuclei around hollow cytoplasmic centers, as occurs with certain other viruses,* was observed.

Occurrence of giant cells was not influenced either in frequency or in rapidity of formation by substitution for the usual culture medium of a medium supplemented with 30 mg% glutamine, or by other media such as Enders' amniotic fluid medium.

Discussion. Presence of inclusion bodies in a cell has long been considered an essential sign of virus infection, also for herpes virus. Consequently efforts were directed to discover in specific inclusion bodies centers of virus formation. Melnick and Reissig(8) demonstrated, however, that no identity nor straight correlation exists between intranuclear herpes inclusion body and accumulation of virus particles. These authors claimed further that appearance of herpes inclusion is dependent on fixative employed. We were able to substantiate this claim only partially. According to our observations an optical densification regular in shape or slightly lobulated appeared in the central nuclear area of living infected cells (Fig. 1-3) but this densification never took on the aspect of routinely recognized herpes inclusion body, sharply delineated and separated from the cell wall by a clear halo. This kind of formation, seen only after fixation and staining, is possibly due to a modification of the physico-chemical state of the nucleoplasm. Both the initial heightened intensity and later nearly complete arrest of Brownian movement in the nucleoplasm, as well-known changes in stain affinities(9) are all signs of profound reorganization of the nucleoplasm as a whole during the infectious process.

Consequently, the typical herpes inclusion body visible in fixed preparations has to be looked upon as the result of these changes

* Unpublished data.

rather than as a restricted site of virus formation or accumulation. Similarly, the cytoplasmic "central body" we described as a specific lesion in polio virus infected cells(10) proved not to contain any special virus agglomeration.*

Activity of mitochondria and of undulating membranes in cells in advanced stages of nuclear lesions is a striking phenomenon. This continuous functioning of the basic cytoplasmic machinery, during early stages of viral development in the cell, seems to be a common feature of cells infected with many viruses as has been observed with Rous(11), polio(10) and adeno(12) viruses.

Another phenomenon, formation of giant cells in virus-infected tissue cultures, also appears to be a quite general one having been observed in measles, varicella, ectromelia* and monkey foamy viruses among others. However, since it occurs for herpes virus with extreme rapidity, the process could be in this case readily observed and its confluence mechanism definitely established. We could not confirm the observations of Gray *et al.* (13) on amitotic divisions in giant cells. Yet the extremely rapid intrusion of nuclei from outside into a crowded area could easily imitate pictures of amitotic division. Here our observations agree with those of Ross and Orlans(14) McNair Scott(15) and Stoker and Newton(16).

The essential factor influencing formation of giant cells seems to reside in structural changes of the cytoplasm, evidenced by its increased fluidity during early stages of infection. This kind of alterations, as well as previously described changes in the nucleus, visualizes a rather profound and general molecular reorganization of both cyto- and nucleoplasm triggered by virus infection.

Summary. Continuous phase contrast observation and microcinematographic recording of living rabbit kidney cell cultures infected with herpes simplex virus, established the following phenomena: 1. Progressive dissolution and dispersion of nucleoli, loss of nucleoplasmic homogeneity, and appearance of intranuclear densification zone but not of a well delineated inclusion body. 2. Persistent activity of cytoplasmic organelles in cells with severe nuclear lesions. 3. Rapid (2-3 hours) formation of giant cells by cell confluence but not by cell division mechanism, accompanied by signs of cytoplasmic liquefaction. The significance of these data is discussed.

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Influence of Low Dose of 2-Acetylaminofluorene on Liver Tumorigenesis in Mice.* (25043)

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Production of hepatomas and other tumors in mice with 2-acetylaminofluorene (AAF) has been observed by Armstrong and Bonser (1,2) and other investigators(4-6). In these experiments, AAF was fed to adult mice for periods ranging from 25 to 77 wks. It has been observed that tumors are produced more readily in younger than in older mice following exposure to carcinogenic hydrocarbons (5-7) or urethan(8), suggesting that administration of AAF to suckling mice might prove especially effective in carcinogenesis. This was done in our experiment, the result being that hepatoma induction was enhanced in some mice.

Materials and methods. Albino mice, 7 to 8 days of age, and related to strain A/He were fed 3 times weekly by stomach tube with 0.05 ml of methocel-Aerosol OT (dioctyl ester of sodium sulfosuccinic acid) suspension containing 1.4% AAF, for total of 10 feedings. Control suckling mice from different litters were similarly fed a suspension containing no AAF. Following each feeding, the mice were returned to original cages. At about 5 weeks of age, they were weaned and segregated according to sex. During treatment, the mothers were maintained on Purina Lab Chow pellets and continuous supply of tap water. Weanlings received the same diet. The experiment was terminated when the young reached one year of age. These mice, as well as those which died earlier, were autopsied and various organs examined for macroscopic tumors. Tissues were excised and fixed in Tellyesniczky's fluid (20 parts 70% ethyl alcohol, 2 parts formalin, 1 part glacial acetic). Tumors recorded were those visible grossly. However, tumor diagnosis in every instance was based on microscopic examination.

Results. (Table I). Hepatomas were observed frequently among male mice treated with AAF in Group 1. Frequently these tumors were multiple, some mice bearing as many as 18 distinct, elevated nodules/liver. Many hepatomas measured 5 to 10 mm in diameter. Both grossly and microscopically, the hepatomas were similar to those described previously by Andervont and Dunn(9). Although 60% of male mice exposed to AAF bore hepatomas (Group 1), the females proved resistant, only one out of 86 (1%) bearing a hepatoma during comparable period (Group 2). A similar sex difference in incidence of induced hepatomas, many tumors among males but none in females, was reported by Leathem(10) following exposure of mice to semi-purified diet containing AAF. A low incidence or complete lack of hepatomas was observed among male and female mice treated with methocel-Aerosol OT suspension alone (Groups 3, 4) or which received no treatment (Groups 5, 6). While exposure to AAF led to increased incidence of hepatomas in Group 1, this carcinogen appeared to have no significant effect on induction of pulmonary tumors. Thus, essentially the same incidence of pulmonary adenomas was observed whether or not the mice received AAF (Groups 1-6).

AAF was administered to mice during 22 days. This brief yet effective period of treatment is considerably less than those periods employed by others(3-6). Also, in the latter experiments(3-6), the compound was given 3 times weekly or continuously, for 25-77 wks. Actual total intake of AAF/mouse is not indicated. However, from concentrations employed and duration of treatment, it is probable that the amounts were many times greater than the 7 mg total dose which each mouse received in present investigation. This 7 mg dose represents to date the smallest quantity of AAF which increased significantly the incidence of hepatomas in mice. However, since this dosage and the 22-day period

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TABLE I. Tumors Induced in Albino Mice with 2-Acetylaminofluorene (AAF), 6 Groups.

Treatment	Total No. mice*	Sex	Observation period (avg days)	Mice with tumors	
				Hepatomas	Pulmonary adenomas
AAF - suspension	62	♂	365	37	17
Idem	86	♀	362	1	27
Suspension only	27	♂	364	1	8
Idem	25	♀	366	0	9
None	29	♂	365	1	10
"	28	♀	365	0	9

* Survivors at time first hepatoma was observed.

of treatment were selected arbitrarily, it is possible that an even smaller amount of AAF, and given over a shorter time, also may be effective. These results demonstrate that 7-8 day old male mouse is highly susceptible to hepatocarcinogenic action of orally administered AAF.

Summary. Administration by stomach tube of a suspension containing 2-acetylaminofluorene to suckling albino mice was followed by development of high incidence of hepatomas in males (60%) but not in females (1%). A similarly low incidence of hepatomas (0%-4%) was observed in untreated mice from same strain or mice treated with suspension alone. The low dose of 2-acetylaminofluorene represents smallest quantity of this compound effective to date in liver tumorigenesis. Also, results indicate that suckling male mouse is especially susceptible to hepatoma induction with 2-acetylaminofluorene.

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Transamination in *Leptospira biflexa.** (25044)

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Few studies on metabolism of leptospirae have been published. Chang(1) reported that sugars were not utilized by *Leptospira icterohemorrhagiae.* Marshall(2) and Helprin and Hiatt(3) studied the effect of serum or fatty acids on oxygen utilization by whole cells of *L. icterohemorrhagiae.* Fulton and Spooner (4) using the same organism analyzed the growth medium for chemical changes and found that small quantities of volatile fatty acids accumulated. An investigation of amino acid metabolism in the leptospirae was undertaken since sugars are not utilized(1). Enzymatic transamination, firmly established in animal tissue and other bacteria(5), was performed with whole cells and cell-free extracts of *L. biflexa.*

Materials and methods. The culture of *L. biflexa* was obtained from A. D. Alexander, Walter Reed Army Medical Center. Leptospirae were grown in 4 l quantities in medium of Cox(6) and harvested by centrifugation

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TABLE I. Transaminations by Cell-Free Extracts of *L. biflexa.*

	mg dry cell ext./ml	Amino donor,† μmoles/ml		Amino acceptor, μmoles/ml	Amino acid formed, μmoles/ml
(a)				α-ketoglutarate	Glutamic acid
	5.8*	None	0	0	0
	"	"	0	16.6	0
	"	DL-Aspartic	16.6	"	1.70
	"	" -Isoleucine	"	"	2.20
	"	" -Leucine	"	"	4.78
	"	" -Methionine	"	"	2.38
	"	" -Phenylalanine	"	"	4.21
	"	" -Tryptophan	"	"	3.49
	"	L -Tyrosine	8.3	"	1.05
	"	" -Valine	"	"	.71
(b)				Pyruvate	Alanine
	4.1	None	0	0	0
	"	"	0	18.2	0
	"	DL-Isoleucine	18.2	"	2.92
	"	" -Leucine	"	"	1.46
	"	L -Glutamic	9.1	"	3.93
(c)				Oxalacetate	Aspartic acid
	5.7	None	0	0	0
	"	"	0	21.7	0
	"	L-Glutamic	10.9	"	6.24

* In all cases the numbers listed represent final concentration.
† Only amino acids showing transamination listed. For those used see text.

after 9 to 12 days incubation at 30°C. Cells were washed 3 times in 0.1 M phosphate buffered saline (pH 7.2) and suspended finally in the same menstruum. Cell-free extracts were prepared by sonic oscillation and freed of cellular debris by centrifugation at 15,000 x G for 15 minutes. Dry weights of cell-free extracts varied between 10 and 20 mg/ml. Cell-free extracts were dialyzed against 10^{-5} M phosphate buffer (pH 7.2) for 24 hours before use to remove glutamic acid present in small quantities. L or DL amino acids were used as amino donors and concentrations of amino acids were doubled when DL mixtures were used. The amino acids tested for transamination included DL-alanine, DL-aspartate, DL-arginine, L-glutamate, glycine, L-histidine, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, and L-valine. In experiments in which glutamate, aspartate, or alanine was produced by transamination, the respective amino acid was not included as amino donor. Keto acid, amino acid, cells or dialyzed cell-free extract, and phosphate buffer (pH 7.2, 0.01 M final concentration) were incubated in test tubes under anaerobic conditions for 150 minutes at 30°C. Reactions were terminated by placing tubes in boiling water bath 5 minutes and removing precipitated cells or protein by centrifugation. Ascending paper chromatography in conjunction with colorimetric procedure of Housewright and Thorne(7) was used to measure quantity of amino acid produced.

Results. A preliminary experiment was performed using α-ketoglutarate as amino acceptor in presence of whole cells. DL-aspartate, DL-isoleucine, DL-leucine, DL-methionine, DL-phenylalanine, DL-tryptophan, L-tyrosine, and L-valine transaminated with α-ketoglutarate to form glutamate.

Since transamination reactions were catalyzed by whole cells, further experiments were done with dialyzed cell-free extracts. Table I shows results obtained with α-ketoglutarate, pyruvate, or oxalacetate as amino acceptors. Absence of amino acid formation in controls demonstrates that amino acids were not produced by non-specific means. Eight amino acids transaminated with α-ketoglutarate, 3 with pyruvate and only glutamate with oxalacetate.

Table II presents results of experiment performed to demonstrate the role of pyridoxal

TABLE II. Effect of Dialysis on Glutamic-Aspartic Transaminase.

Additions incubated at 30°C for 70 min.		Glutamate formed, μmoles/ml
Aspartate	18.5 μmoles/ml*	2.21
α-ketoglutarate	*Idem*	
Undialyzed cell-free ext.	8.8 mg/ml	
Aspartate	16.6 μmoles/ml	1.70
α-ketoglutarate	*Idem*	
1×10^{-5} M phosphate buffer dialyzed cell-free ext.	5.8 mg/ml	
Aspartate	18.5 μmoles/ml	0
α-ketoglutarate	*Idem*	
Versene dialyzed cell-free ext.	4 mg/ml	
Aspartate	15.6 μmoles/ml	.51
α-ketoglutarate	*Idem*	
Versene dialyzed cell-free ext.	4 mg/ml	
Pyridoxal phosphate	12.5 "	

* In all cases the numbers listed represent final concentration.

phosphate in transamination reactions of *L. biflexa*. Dialysis of cell-free extract against 10^{-5} M phosphate buffer (pH 7.2) even if extended over a long period of time did not eliminate aspartic-glutamic activity. Dialysis of cell-free extracts against versene according to procedure of Racker, *et al.*(8), followed by dialysis against distilled water to remove versene, resulted in complete loss of transamination activity. Activity was partially restored by addition of pyridoxal phosphate.

Discussion. Whole cells and cell-free extracts gave similar results when α-ketoglutarate was the amino acceptor. Leptospirae require oxygen for growth and do not utilize sugars. Consequently, no apparent means of energy metabolism transpires to facilitate transportation of keto acid or amino acid into the cell. It seems unlikely that both amino acid and keto acid are passively permeable.

In general, transamination reactions of *L. biflexa* are similar to those known to occur in other microorganisms, plant and animal tissues(5). However, different amino acids are active in leptospiral transamination in comparison to reactions in another spirochete, the Reiter treponeme(9). The capacity of cell-free extracts of *L. biflexa* to carry out a number of transamination reactions emphasizes an active amino acid metabolic system whether or not such reactions are involved in synthesis or degradation. That leptospirae possess cytochrome(4), and are able to metabolize amino acids to some extent as shown here, indicates that amino acids should be considered as possible energy sources along with fatty acids(3). Further studies on oxidative deamination of amino acids by leptospirae and determination of citric acid cycle enzymes would be very important in this regard.

Most reactions studied were not checked for reversibility. However, the glutamic-aspartic reaction was reversible. Attempts to demonstrate reversibility of glutamic-alanine transamination reaction were not successful, although several experiments were performed with varying concentrations of alanine. We can give no explanation of these results.

That pyridoxal phosphate is the coenzyme for transamination was expected. It is of interest that dialysis against versene resolved the system. Perhaps greater reactivation could have been attained by addition of magnesium ions along with pyridoxal phosphate (10).

Summary. Whole cells and cell-free extracts of *Leptospira biflexa* have been used to demonstrate a series of transamination reactions. Dialysis against versene was used to show that pyridoxal phosphate served as a coenzyme. The possible importance of transamination in leptospiral metabolism is discussed.

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Purine Analogs as Feedback Inhibitors.*† (25045)

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A feedback mechanism of biosynthetic control which shuts down formation of purine precursors has been described for auxotrophic mutants of *Escherichia coli* that accumulate the substrate of their genetically blocked reaction(2,3). One such mutant, strain B-96, is lacking in transformylase activity and hence accumulates the ribotide of 5-amino-4-imidazolecarboxamide (AICAR) which is dephosphorylated in the process of its excretion and appears in the culture fluid as riboside form. Accumulation of AICAR comes to an immediate halt when minute amounts of any purine which can support growth of mutants are added to the medium. Thus, a direct correlation exists between those purines which can be used for nucleic acid synthesis and those which are able to trigger the shut-down mechanism of biosynthetic control. For a clearer insight into the action of purines as feedback inhibitors, it became desirable to find substances which could prevent this inhibitory action. The "antipurines," inhibitory structural analogs of naturally-occurring purines, appeared to be likely candidates for such a role. A number of antipurines were tested, but instead of nullifying feed-back inhibition, those which were active simulated natural purines as potent inhibitors of AICAR formation. This suggested that the structural similarities which made them competitive antimetabolites were sufficient to mimic the natural purines in activating feedback mechanisms of control. This report deals with analysis and description of effects of purine analogs on formation of the purine precursor, AICAR.

Materials and methods. *Escherichia coli,* strain B-96, is a purine-requiring mutant isolated in this laboratory from the wild-type, strain B, by penicillin-selection technic. It is non-discriminating in its choice of purines for growth. The accumulation of AICA (as the riboside) was observed in non-proliferating suspensions of bacteria by methods previously described(4). Non-proliferating cell suspensions (0.5 mg dry weight/ml) were incubated in phosphate buffer (0.04 M at pH 7.4) containing $MgSO_4$ (0.01%), glucose (0.02 M) as source of carbon, and ammonium chloride (0.1%) as source of nitrogen. Casein hydrolysate (0.1%) was also added to increase the yield of AICA. At appropriate time intervals, aliquots were removed, cells were precipitated with trichloroacetic acid and AICA content of supernates was measured as diazotizable amine by the method of Bratton and Marshall(5).

Results. The amount required for 50% inhibition of AICA formation was determined for 15 analogs and related compounds (Table I). The most active inhibitors were the 3 antimetabolites which have gained considerable popularity as growth inhibitors in many other systems, *viz.*, 6-thioguanine, 6-mercaptopurine and 2,6-diaminopurine. As little as 1 μg of these compounds/ml of test system reduced the yield of AICA by at least 50%. The 8-azapurines were comparatively less active as inhibitors.

Dose response curves are shown in Fig. 1. It is evident from these curves that a proportionality exists between degree of inhibition and logarithm of concentration of the antimetabolites. With increased concentrations, a plateau level of maximal inhibition was reached at 70 to 85%; complete inhibition was never achieved. The kinetics of inhibition are shown in Fig. 2 with 6-mercaptopurine as an example. With 6-mercaptopurine present from the beginning, rate of formation of AICA comes to nearly a complete halt after 30 minutes. When addition of the antimetabolite is delayed, the decelerative action becomes more pronounced. After production of AICA has been under way for one hour, addition of 6-mercaptopurine causes immediate and complete cessation of further formation. This

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TABLE I. Effect of Purine Analogs on Formation of AICAR by *Escherichia coli*, Strain B-96.

Compound*	Purine substituents 2	6	8	Other	Amt for 50% inhibition (μg/ml)
6-thioguanine	NH_2	SH			.2
6-mercaptopurine		SH			.5
2,6-diaminopurine	NH_2	NH_2			1.0
8-aza-adenine		NH_2	(—N=)†		20.0
8-azaguanine	NH_2	OH	(")		100
8-azahypoxanthine		OH	(")		"
Pyrazoloadenine		NH_2	(")	7: (—CH=)	"
2-thioxanthine	SH	OH			160
8-azadiaminopurine	NH_2	NH_2	(")		200
Kinetin		NHC_4H_3O			500
8-azaxanthine	OH	OH	(")		700
2-thio-8-azaxanthine	SH	OH	(")		>1000
2-thioadenine	SH	NH_2			"
Benzimidazole				1,3: (—CH=)	"
Puromycin riboside		$N(CH_3)_2$		9: 3′-NH_2-ribose	"

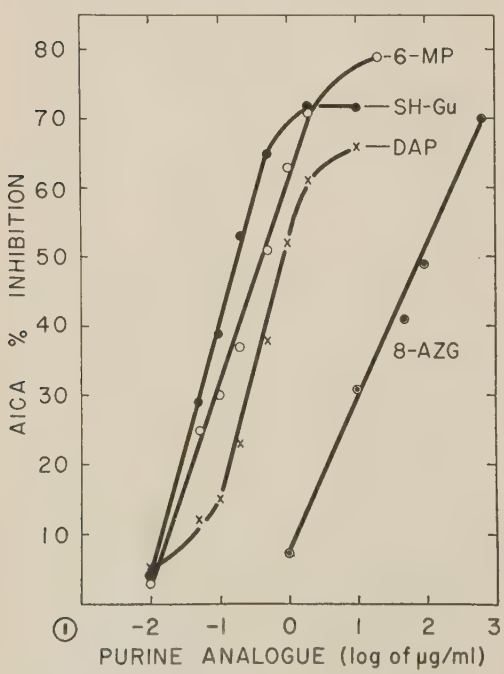
* We are particularly grateful to Sigma Chemical Co. and to Dr. G. H. Hitchings of Wellcome Research Labs for generous gifts of a number of compounds; and to Dr. J. R. Thomson of Southern Research Inst. for sample of 4-aminopyrazolopyrimidine (pyrazoloadenine).

† Symbols in parentheses refer to substitutions within the purine ring, *e.g.*, 8: (—N=) means that the —CH= at position 8 has been replaced with —N=; 7: (—CH=) means that the —N= at position 7 has been replaced with —CH=.

Yield of AICA was determined after 3 hr of incubation at 37°C.

experiment not only shows direct and immediate inhibitory action of 6-mercaptopurine but also serves to rule out the possibility that inhibition may have been at the level of enzyme formation. The effects observed are strikingly similar to those obtained with adenine as a feedback inhibitor (3).

When the analogs are classified as to their



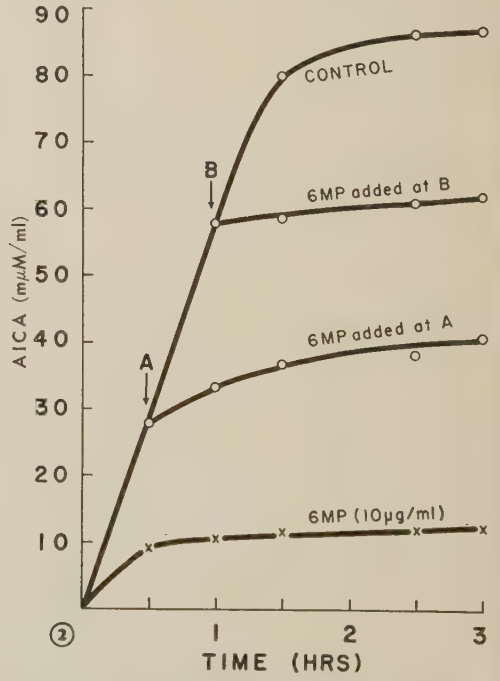


FIG. 1. Dose response curves of various purine analogs as inhibitors of AICA accumulation by *E. coli*, strain B-96. 6-mercaptopurine (6-MP); 6-thioguanine (SH-Gu); 2,6-diaminopurine (DAP); 8-azaguanine (8-AZG).

FIG. 2. Inhibition of AICA accumulation by 6-mercaptopurine (6-MP). Additions of 6-MP to give final concentration of 10 μg/ml were made as indicated.

effectiveness as inhibitors of bacterial growth, this is seen to correlate with their effectiveness as inhibitors of AICAR formation. The best inhibitors of growth are those which are potent inhibitors of AICAR formation; those which are inactive as inhibitors of biosynthesis are also inactive as inhibitors of growth. No simple quantitative correlation was found. For example, 2,6-diaminopurine, which gives a half-maximal inhibition of AICA formation at 1 μg/ml, requires 10 times this concentration to give half-maximal inhibition of growth. The quantitative discrepancies may reside partially in the fact that complete inhibition of AICAR formation is never obtained and partially in the possibility that metabolic diversions of analogs may occur during growth.

Discussion. In addition to the traditional action of successful antipurines as competitive inhibitors at the level of interconversions and utilization of nucleic acid purines, apparently at the nucleotide level, it is becoming increasingly evident that they may also inhibit at the level of *de novo* biosynthesis of nucleotides. Incorporation of isotopically labeled glycine into nucleic acid purines of tumor cells is inhibited by 2,6-diaminopurine and, to a lesser extent, by 6-mercaptopurine(6). A multi-site inhibitory action of 6-thioguanine has also been revealed in metabolism of purines by tumor cells, with inhibition of *de novo* biosynthesis occurring before formation of the precyclic precursor, formylglycinamide ribotide (7). Inhibition at this level would prevent formation of the cyclic aminoimidazole precursor studied here. It is suggested that inhibitory antipurines act as sequential inhibitors by preventing both biosynthesis and eventual metabolic utilization of purine nucleotides. Indeed, those which are effective chemotherapeutic agents may owe their success to this sequential blockade.

The results reported here suggest that the very property of structural similarity which endows the analogs with competitive action may also allow them to trigger the feedback mechanism of biosynthetic control. This is particularly evident for those analogs which prevent formation of AICA at levels of 0.01 micromole or less. For those requiring more than 1 micromole for effective inhibition, as with most 8-azapurines, a pseudo-type of feedback inhibition may be operating. This could occur by limiting availability of 5-phosphoribosyl-1-pyrophosphate (PRPP) for *de novo* biosynthesis. The utilization of PRPP in formation of analog-ribonucleotides has been reported for a variety of purine analogs(8,9). Final evaluation of the mechanism of action of antipurines, particularly as inhibitors of *de novo* synthesis, must also take into account possible competition with purine-containing cofactors which participate in the biosynthetic reactions.

Summary. Accumulation of ribotide of 5-amino-4-imidazole carboxamide (AICAR), excreted in the riboside form by a purine-requiring mutant of *Escherichia coli,* was inhibited by a number of structural analogs of purines. The most potent inhibitors were 6-thioguanine, 6-mercaptopurine, and 2,6-diaminopurine. The type of inhibition obtained suggests that the antimetabolites sufficiently resemble natural purines in structure to act as feedback inhibitors in biosynthetic control.

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Plasma Lactic Dehydrogenase Activity in Experimental Hemorrhagic Shock.* (25046)

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Recent reports describe increased serum lactic dehydrogenase (LDH) activity in several disease states which affect primarily individual tissues(1,2,3). This study was undertaken to determine whether widespread damage produced by hemorrhagic shock would result in elevations of plasma LDH activity.

Materials and methods. A. *Hemorrhagic shock in dogs.* Ten healthy mongrel dogs (average weight 20 kg), premedicated with morphine† (1-2 mg/kg intramuscularly), were bled from femoral artery into an elevated reservoir in parallel with a mercury manometer (4). The artery was cannulated under local anesthesia. Heparin, 40 mg, was administered to prevent clotting Height of reservoir was adjusted so that arterial pressure was 30 mm Hg in 6 dogs, and 40 to 50 mm Hg in 4 dogs. The animals were maintained at these pressures until death. Four ml of blood for LDH assay was obtained at intervals of 1 to 2 hours by needle puncture from uncannulated femoral artery. Eight additional dogs similarly prepared were transfused with all blood in reservoir after 1 to 5 hours of hemorrhagic hypotension. Arterial pressure was maintained at 30 mm Hg until transfusion. Arterial blood was obtained at intervals for assay of plasma LDH. Arterio-venous differences in plasma LDH activity were determined in 3 dogs. Catheters were placed in hepatic and renal vein *via* jugular and femoral vein respectively. Placement was assured by laparotomy under pentobarbital anesthesia. After closure of abdomen and recovery from anesthesia animals were subjected to hemorrhagic hypotension at arterial pressure 30 mm Hg. Simultaneous samples were drawn before hemorrhage and at intervals thereafter from the hepatic, renal and femoral veins and femoral artery until death. Post-mortem examination revealed that catheters had remained in place during experiments. B. *Hemorrhagic shock in rabbits.* Six adult albino rabbits (average weight 2 kg) were subjected to hemorrhagic shock by same technic. Arterial pressure was kept at 50 mm Hg. Arterial samples for LDH assay were taken before shock and at intervals until death. C. *Lactic dehydrogenase assay.* Blood specimens were centrifuged immediately. Plasma was kept at 4°C for less than 24 hours, then analyzed spectrophotometrically for LDH activity by the method of Wróblewski and LaDue (1). One unit of activity was defined as a decrease in optical density of 0.001/ml of plasma on scale of Beckman DU spectrophotometer using 1 cm path length at wave length of 340 mu. Since red cells exhibit much greater LDH activity than plasma(1), the extent of hemolysis was determined. Plasma hemoglobin concentrations of dogs in shock, determined by modified dibenzidine method (5), indicated no significant contribution of hemolysis to the observed elevations in LDH activity. Electrophoretic separation of dog plasma obtained before and during shock was performed on starch supporting medium and the eluates of half inch starch segments were assayed for LDH activity(6). Protein was determined by modification of Folin-Ciocalteau procedure(6). A water flow experiment revealed no differential absorption of LDH activity by starch. D. *Other enzymes in shock.* Simultaneous measurements of activity of leucine aminopeptidase(7), alpha-glucosidase(8), and beta-glucuronidase(9) were made in plasma of 2 dogs during hemorrhagic shock. Ceruloplasmin(10) and glutamic oxalacetic transaminase (GO-T)(11) were assayed in 3 dogs, and malic dehydrogenase (MDH)(3) and alkaline phosphatase(12) in three.

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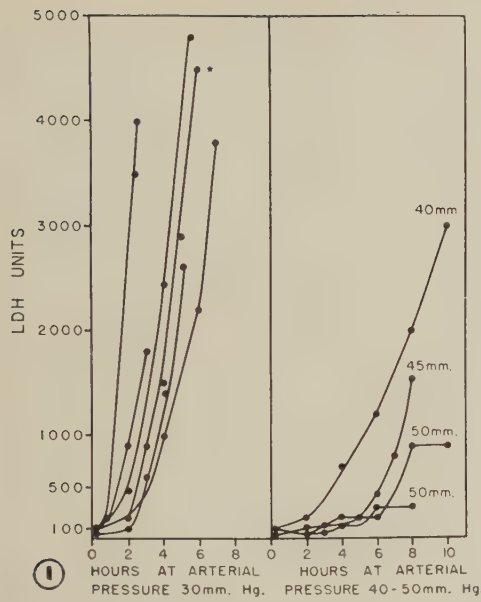
† This dose of morphine does not produce increases in plasma LDH activity.



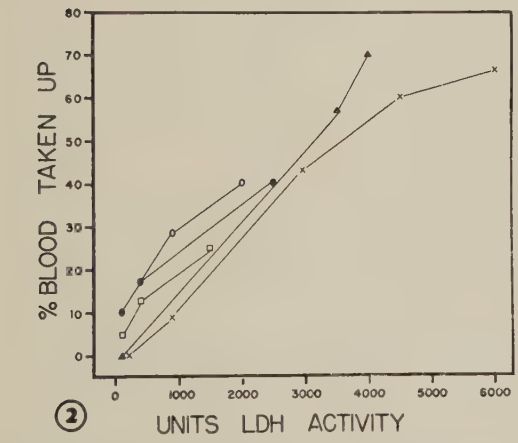


FIG. 1. Plasma LDH activity of 10 dogs during sustained hemorrhagic hypotension. Curve marked with asterisk went on to 16,000 units before death at 9 hr.

FIG. 2. Relationship between spontaneous withdrawal of blood from the reservoir ("taking up") and plasma LDH activity in 5 dogs maintained at arterial pressure 30 mm Hg.

Results. A. *Hemorrhagic shock in dogs.* Plasma LDH activity of 6 dogs maintained at arterial pressure of 30 mm Hg showed initial lag period of 1 to 3 hours followed by sharp rise (Fig. 1). The activity rose from a mean of 75 units before shock to 1000 to 4000 units in all animals at this pressure for more than 2 hours.

In 4 dogs maintained at arterial pressure 40 to 50 mm Hg the lag period was longer, 4 to 6 hours, and elevations immediately thereafter were more gradual. As these animals deteriorated, LDH activity increased markedly so that slope of curve terminally resembled that of dogs bled to arterial pressure 30 mm Hg.

Animals maintained at constant hypotensive levels by elevated reservoir technic eventually exhibit spontaneous return of blood from reservoir, a response called "taking up" (13). Fig. 2 reveals a direct linear relationship between plasma LDH activity and rate of "taking up." To determine whether LDH activity was affected by blood returning from reservoir, samples of reservoir blood throughout hemorrhagic shock were assayed and no change in plasma LDH activity occurred. Furthermore, when a liter of blood permitted to stand *in vitro* for 8 hours was infused by single exchange transfusion into a normal dog, no alteration in LDH activity of recipient occurred.

Of 8 additional dogs bled to an arterial pressure of 30 mm Hg for 1 to 5 hours and then transfused with all blood remaining in reservoir, the 3 animals that died showed progressive increases in plasma LDH activity after transfusion, reaching 90 to 200 times initial values (Fig. 3). These animals exhibited the course and post-mortem findings characteristic of irreversible hemorrhagic

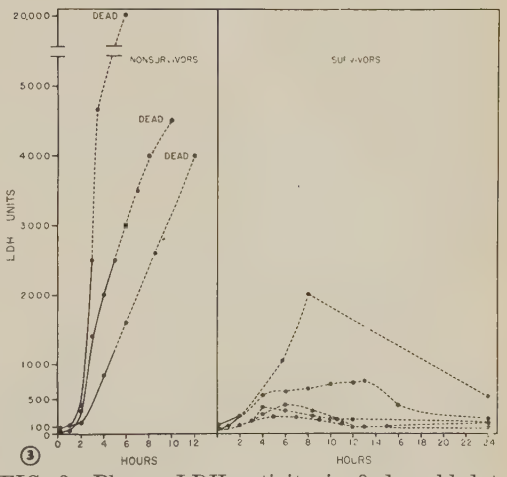


FIG. 3. Plasma LDH activity in 8 dogs bled to arterial pressure 30 mm Hg for 1, 2, 3, and 5 hr and then given replacement transfusion. Solid lines show activities during hemorrhagic hypotension, broken lines after transfusion.

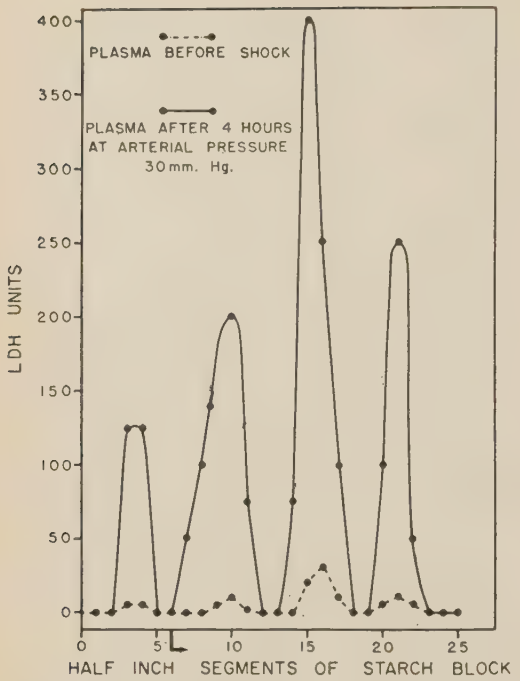


FIG. 4. Electrophoretic pattern of LDH activity in dog plasma before and during hemorrhagic hypotension. Four peaks of LDH activity are present. From the left these are located in gamma globulin, beta globulin, alpha-2 globulin and between alpha-1 globulin and albumin. All are elevated in shock. The origin is at the arrow.

shock. The 5 survivors, bled 1 to 2 hours, showed a post-transfusion rise of 3 to 25 times the initial value. These elevations were sustained for 6 to 12 hours and receded toward normal within 24 hours.

Plasmas from 2 dogs in hemorrhagic shock were fractionated electrophoretically. Fig. 4 shows distribution of plasma LDH activity before and during shock in one animal. Four peaks are present and located in the gamma globulin, beta globulin, alpha-2 globulin, and between the alpha-1 globulin and albumin. All peaks are elevated in shock plasma. Findings in a second experiment were similar.

In all 3 experiments in which the A-V difference in LDH activity was measured, plasma LDH activity in hepatic, renal and femoral venous blood attained consistently higher levels than that of arterial blood. Maximum differences were 200 units in renal and hepatic venous blood and 100 units in femoral venous blood.

B. *Hemorrhagic shock in rabbits.* In 6 experiments plasma LDH activity increased from initial values below 150 units to 1800 to 3200 units within 10 hours. An initial lag period of 2 to 4 hours was observed.

C. *Other enzymes in shock.* Elevations of 3 other enzymes, a dehydrogenase (malic), a transaminase (glutamic oxalacetic) and a phosphatase (alkaline) were observed during hemorrhagic hypotension at 30 mm Hg. In 3 experiments simultaneous determinations of LDH, MDH and glutamic oxalacetic transaminase (GO-T) revealed that the pattern of alterations of these 3 enzyme activities was very similar. Elevations of 20 to 40 times prehemorrhagic values appeared within 8 hours. Another experiment showed elevations of alkaline phosphatase activity which reached 10 times prehemorrhagic values within 7 hours. In all cases a lag phase of 2 hours preceded the elevations.

A number of enzymes assayed simultaneously during sustained hemorrhagic hypotension did not show significant alteration in activity. These include leucine aminopeptidase, alpha-glucosidase, beta-glucuronidase, and ceruloplasmin.

Discussion. These experiments reveal a marked elevation of LDH activity in plasma of dogs and rabbits subjected to hemorrhagic shock. The significance of alterations of this enzyme in shock lies in the relationship these alterations bear to survival or death of an animal. Duration of the lag phase of the activity curve is about equal to duration of hemorrhagic hypotension which most animals will survive if given replacement transfusion.‡ In animals that do not survive hemorrhagic hypotension, whether transfused or not, LDH activity invariably continues to rise until death. In animals that do survive hemorrhagic hypotension, LDH activity continues to rise for several hours after replacement transfusion, but thereafter recedes toward normal. This suggests that a declining curve of LDH activity is a favorable prognostic sign.

Alterations in plasma LDH activity in

‡ Survival is usual if animal is transfused within 2 hours. If transfused after 4-5 hours death is the rule.

hemorrhagic shock coincide with certain other parameters of biological deterioration. Fig. 2 shows a linear relationship between LDH activity and rate and extent of "taking up," an important index of survival(13). The greater the percentage of take up, the less likely is the dog to survive. That the curve of LDH activity reflects extent of trauma imposed is indicated by longer lag period and more gradual elevation of activity in dogs maintained at arterial pressures of 40, 45, and 50 mm Hg as compared to those at 30 mm Hg (Fig. 1).

Since present methods measure LDH activity, not concentration, some factors which affect activity were considered. An inhibitor of LDH activity has been described by Greig in livers of dogs in hemorrhagic hypotension (14). When we mixed shock plasmas of high and low LDH activities, the mean values obtained suggested that an activator or inhibitor substance in plasma was not present. Nor was an activator found in reservoir blood. Alterations in pH are not a likely explanation of the rise in LDH activity, for the pH optima for LDH are alkaline(15) whereas in shock the pH falls. Nor is elevation in activity attributable to concentration of serum proteins, since these do not change significantly(16). Increase in blood pyruvate which occurs in hemorrhagic shock(16) was not permitted to influence measurement of LDH activity, for the pyruvate was removed by incubation prior to LDH assay(1).

The higher LDH activities in hepatic, renal and femoral vein blood compared to that in arterial blood indicate that many tissues may contribute to the elevation in plasma activity. These results are in harmony with a report showing appreciably reduced GO-T activities in homogenates of skeletal muscle, kidney, liver and cardiac muscle in rats after severe anoxia(17).

Starch zone electrophoresis has been used to investigate homogeneity of lactic dehydrogenase. Results indicate that LDH is electrophoretically heterogeneous(18) and that more than one enzyme with LDH activity is present in each of several mammalian tissues(19). The finding of 4 enzymes in dog plasma is in contrast to the 3 enzymes in human plasma and to the 2 in rabbit plasma(15,18). Of the 3 in human plasma, one is elevated in myocardial infarction and a different one in leukemia (15,18,20). In shock in dogs all 4 enzymes are elevated. If these enzymes are derived from different tissues, the elevation of all peaks may be taken to reflect known widespread damage in shock.

Summary. 1. Marked elevations of plasma LDH activity in experimental hemorrhagic shock are described. An initial lag period is followed by a sharp rise to 15 to 50 times the original value. 2. Alterations of plasma LDH activity in shock bear a consistent relationship to such parameters of biological deterioration as rate of spontaneous return of blood from the reservoir to the animal ("taking up") and reversibility to replacement transfusion. 3. Catheterization experiments in which consistent arterio-venous differences were demonstrated indicate that the sources of elevated plasma LDH activity are widespread and include liver, kidney and tissues of an extremity. 4. Electrophoretic separation of normal dog plasma reveals 4 enzymes with LDH activity. All 4 are elevated in plasma of dogs in shock. 5. Marked elevations of plasma GO-T, MDH and alkaline phosphatase were observed in dog plasma during hemorrhagic hypotension. No significant elevations in beta-glucuronidase, alpha-glucosidase, leucine aminopeptidase and ceruloplasmin occurred. 6. These experiments suggest that serial plasma LDH determinations may serve as useful indicators of the extent of biological deterioration in hemorrhagic shock.

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Action of Semen Inducing Rupture of Yolk Membrane in Chicken Egg. (25047)

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Pincus(1) and Yamane(2) simultaneously reported that cumulus cells which surround the eggs of rabbits were diffused by adding their semen. The latter concluded that 2nd maturation division of the ovum is dependent on penetration of spermatozoon and that it also can be artificially induced by treating with pancreatin. Recently in experiments on fertilization we found that the yolk membrane of hen's eggs was ruptured on addition of semen. These tests were made *in vitro* where artificially ovulated eggs were used. The present study was made to analyze abovementioned phenomenon.

Methods. Newly laid eggs and ova artificially ovulated *in vitro* according to methods described by Neher, Olsen and Fraps(3) and Saeki and Katsuragi(4) were used. Semen collected from the animal was placed in contact with the yolk membrane of eggs kept in porcelain dishes and incubated at 38°C. Lengths of time required for semen to rupture the vitelline membrane and release of yolk material were recorded. Hyaluronidase activity of fowl semen was estimated by decreasing the viscosity of hyaluronic acid solutions (a modification of McClean's method) (5) and by increasing the area of spread of intradermally injected substances. Purified hyaluronidase "Sprase" of Mochida Pharma. Mfg. Co., Tokyo, was used.

Results. Effect of Semen of Different Animals on Yolk Membrane. Rupture of yolk membrane of hen's eggs is brought about by semen from cocks and from other domestic animals. Length of time required for rupturing the membrane after adding semen is shown in Table I. Vitelline membrane of eggs serving as controls where semen was not added, was kept intact during first 5 days in the following experiment (Table I). The vitelline membrane of artificially ovulated ova was ruptured within 2 hours by cock's semen. The same semen required 6.3 hours to rupture the yolk membrane of normally laid egg. It seemed strange that semen of rabbits, although unrelated to Aves, was more effective than semen from the duck, which is in the same class with the domestic fowl. Boar's semen was least effective.

Action of spermatozoa and semen serum. Spermatozoa were separated from seminal fluid by centrifuging the semen 20 minutes at 20,000 RPM, then both fractions were placed separately on the vitelline membrane of egg yolks. As shown in Table II, the destructive action of spermatozoa was higher than that of semen serum. This suggests that the former contains more effective substance(s) than the latter.

TABLE I. Effect of Adding Animals' Semen on Rupture of Yolk Membrane of Chicken Egg.

Semen collected from	Exp. No. of eggs used	Exp. cc semen added	Exp. Times required for rupturing Hr	Exp. Min.	Exp. Range	Control
Cock	10*	.2	1	53	1:00– 3:30	Untreated 35 eggs did not rupture during first 5 days
"	68	.1	6	18	2:00–22:10	
Drake	15	.1	9	28	7:10–20:00	
Rabbit	25	.1	4	37	0:30– 9:15	
Goat	13	.5	20	47	10:45–48:00	
	8	.1	Not rupt.†			
Bull	17	.5	18	38	10:20–46:20	
	8	.2	Not rupt.†			
Boar	25	.5	39	00		
	8	.1	Not rupt.†			

* These are ovulated ova *in vitro* from excised follicles.
† These did not rupture during first 5 days.

Effect of heat on semen. Semen was heated at 65, 70, 75, 80, 97 and 100°C for 10-20 minutes to test its heat resistance. Semen heated above 70°C took longer to rupture the vitelline membrane. In 11 of 15 samples heated at 100°C for 20 minutes, the destructivity was retained for as long as 28 hrs, indicating that the destructive principle(s) present in semen possess(es) to some extent thermal resistance. When rabbit's semen was heated at 80°C and 97°C for 10 minutes, it took 33.75 and 41.7 hours respectively for rupture of yolk membrane of eggs. On the basis of the last experiments, it is suspected that the active principle is a hyaluronidase-like substance.

Measurement of hyaluronidase level and effect of its substance. As far as we know, the hyaluronidase level is very low or absent in cock's semen. Both samples of "semen (0.2 cc) Indian-ink (0.1 cc)" and "Indian-ink (0.3 cc)" were injected into rabbit's derma respectively. Average area of sample containing semen was larger than that of the control, and the level in both samples during 1 hr. was linear. Hyaluronidase level as determined by the viscosimetric method is shown in Table III. Although individual differences exist, average level in cock's semen was remarkably low as compared to that reported in other animals, and it averaged 87.2 viscosity reducing units.

To further ascertain this effect, hyaluronidase (Sprase) extracted from bull testicles was used. The result fell short of our initial assumption, and by using diluted Sprase solution in 525 V.R.U., it took 38.8 hours to rupture the yolk membrane.

Effects of (1) homogenized fluids of various organs and (2) trypsin. The effect of homogenized fluids of various organs of a laying hen and a male chick are shown in Table IV. The solutions prepared from kidney liver and pancreas of laying hens showed nearly the

TABLE II. The Action of Spermatozoa and Semen Serum on the Rupture of the Yolk Membrane.

	No. of eggs	Added cc	Times required for rupturing Hr	Min.	Remarks
Spermatozoa	15	.1	2	52	Semen separated by ultra-centrifuge at 20,000 r.p.m. for 20 min.
Semen serum	15	.1	5	58	
Spermatozoa	15	.1	7	30	Semen separated using centrifuge at 3,000 r.p.m. for 20 min.
Semen serum	12	.1	17	22 (2)	
			Not rupt.	(10)	
	9	.5	12	16	

Note: Ten untreated eggs did not rupture during first 5 days.

TABLE III. Hyaluronidase Level of Cock's Semen Determined by Viscosimetric Technic.

Sperm conc./cc (million)	% surv. sp. and motility	H-ase level/cc (V.R.U.)	Remarks
2.85	100	86.7	Estimated using 0.2% potassium hyaluronate as substrate.
2.98	100	34.9	
2.75	90	131.4	
2.98	100	92.3	
2.95	100	78.0	
2.84	90	150.8	
2.70	100	66.6	
1.97	90	57.1	
Rabbit		3833.5	mean of 4 times

same effect as did their semen. Trypsin, a product of Merck, was strongly effective only in very high concentration, but less effective in 1% solution.

Discussion. The mechanism of fertilization in lower animals, especially in the *sea urchin,* is well known. We thought that the destruction of the yolk membrane in chicken egg on addition of semen might be related to fertilization, and that determination of the chemical substance(s) effecting it might contribute to a higher level of fertility in artificial insemination.

Yamane(1) reported that the proteolytic action of mammalian spermazoa was due probably to a tryptase active in an alkaline medium. However, Huggins and Neal(6) found a little "trypsin" in the semen. Lundquist(7) described a casein-hydrolysing enzyme like "trypsin." Recently, Lundquist *et al.*(8) accomplished the separation of 2 proteolytic enzymes: proteinase and aminopeptidase in human semen.

Furthermore, it is said that the vitelline membrane is composed of keratin and mucin (Morgan and Hall(9)) and collagenous membrane (McNally(10)). Considering these reports, including our data where trypsin was used, there is no denying the fact that proteolytic action participates in the rupture of vitelline membrane.

TABLE IV. Effects of Adding Homogenized Fluid of Various Organs on Rupture of the Yolk Membrane.

	Laying hen		Chick (♂, 2 mo)		
Organs	Hr	Min.	Hr	Min.	Control
Liver	7	44	25	16	10 eggs on which normal semen was added, ruptured during first 7½ hr.
Breast muscle	25	53	25	41	
Kidney	6	35	12	20	
Oviduct	12	25			
Comb	11	36	13	43	
Pancreas	7	41	5	20	
Testis			15	36	

Although it is recognized that differences exist in the action of a purified substance, our experiment using "trypsin" showed unsatisfactory results.

Since we suspected a stronger acting substance, we attempted to measure the hyaluronidase level in cock's semen.

Sweyer(11) and Yasuda and Takahashi (12) reported that fowl semen did not contain hyaluronidase. In our experiment its level was very low when compared with that of other animals. We wish to emphasize however that the destructive action of spermatozoa was stronger than that of seminal fluid and the hyaluronidase extracted from testicles of bulls was rather weak in causing the rupture of vitelline membrane.

The results mentioned above indicate that both trypsin-like substance(3) and hyaluronidase are not very effective in causing rupture of vitelline membrane. Further study to determine the active substance(s) is necessary.

Summary. We found that the vitelline membrane of hen's egg was ruptured on contact with semen. Also that the destructive action was stronger in spermatozoa than in seminal fluid. Experiments were conducted to determine the substance(s) effecting destruction of the membrane. Trypsin and hyaluronidase are responsible for this phenomenon, but another major factor may exist which determines the destruction.

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Labile Phosphate Changes in Ileum of the Rat. (25048)

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Mass movement of large intestine related to eating is a well-known phenomenon. Grossman(1) states that the small intestine also shows increase in motility within a few minutes after eating and suggested the possibility of hormonal involvement. Picchioni and Edwards(2) noted that isolated intestinal strips had greater activity if animals were fed just prior to sacrifice. Kroeger and Edwards(3) reported when rats were fasted to 48 hours and fed just before sacrificing, this treatment caused not only changes in activity but changes in acid-soluble phosphate fractions related to intermediate metabolism and muscle contraction. Whereas changes in phosphate compounds reported by these authors occurred in combined samples of mucosal and muscularis tissue, our object was to determine the extent to which both tissues separately undergo these changes. The data indicate that each tissue shows significant chemical changes with regard to fasting and feeding treatment but that there is no real difference between the 2 tissues.

Methods. Sixty Sprague-Dawley albino rats of either sex and weighing 170 to 275 g, were divided randomly into 6 equal groups. Rats were fed a standard ration of laboratory pellets† except as noted for different groups. Group I was fed *ad lib* until time of sacrifice. Groups II, III and IV were fasted 24, 48 and 72 hours respectively. Animals in Groups V and VI were fasted 48 hours, then allowed to nibble on food pellets for 10 and 15 minutes prior to sacrifice. All groups were given water *ad lib*. The last 24 cm of ileum was removed, washed with ice-cold saline and divided into 4 segments. These segments were opened longitudinally, laid serosa-side down on absorbent toweling and frozen with liquid nitrogen. After mucosa was cut off with razor blade, both mucosal and muscularis samples were refrozen and pulverized in stainless steel mortar. Extraction and assay methods previously reported(3,4) were used and phosphate fractions determined were: T.A.S.P.-P. representing total acid-soluble phosphates, I.P.-P representing ortho phosphate present in tissue as such, "Cr.P.-P." phosphate hydrolyzed at 25°C for 30 minutes in 1 N.H_2SO_4 and "A.T.P.-P.", the phosphate hydrolyzed at 100°C for 20 minutes in 1 N.H_2SO_4.

Results. Concentrations of various phosphate fractions expressed in mg% phosphorus/wet weight tissue are shown in Fig. 1, 2, 3, and 4, each value representing 40 assays. Analysis of variance and the Mann-Whitney "U" test(5) for ranked data indicates that treatments are significant at least to 0.05 level of confidence. Variation between mucosal and muscularis samples are not significant. All phosphate fractions show a decline in median concentration as period of fasting is extended except A.T.P.P. in mucosal segments. This increases slightly during first 24 hours but is comparable with the muscularis tissue at 72-hour period. This rise was previously noted(3) using combined samples of mucosa and muscularis.

Most concentrations for different fractions decrease gradually, reaching 50 to 60% of control values by 72-hour period. As fasting period is prolonged all phosphate fractions become more uniform in range of concentra-

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† Purina Lab Chow

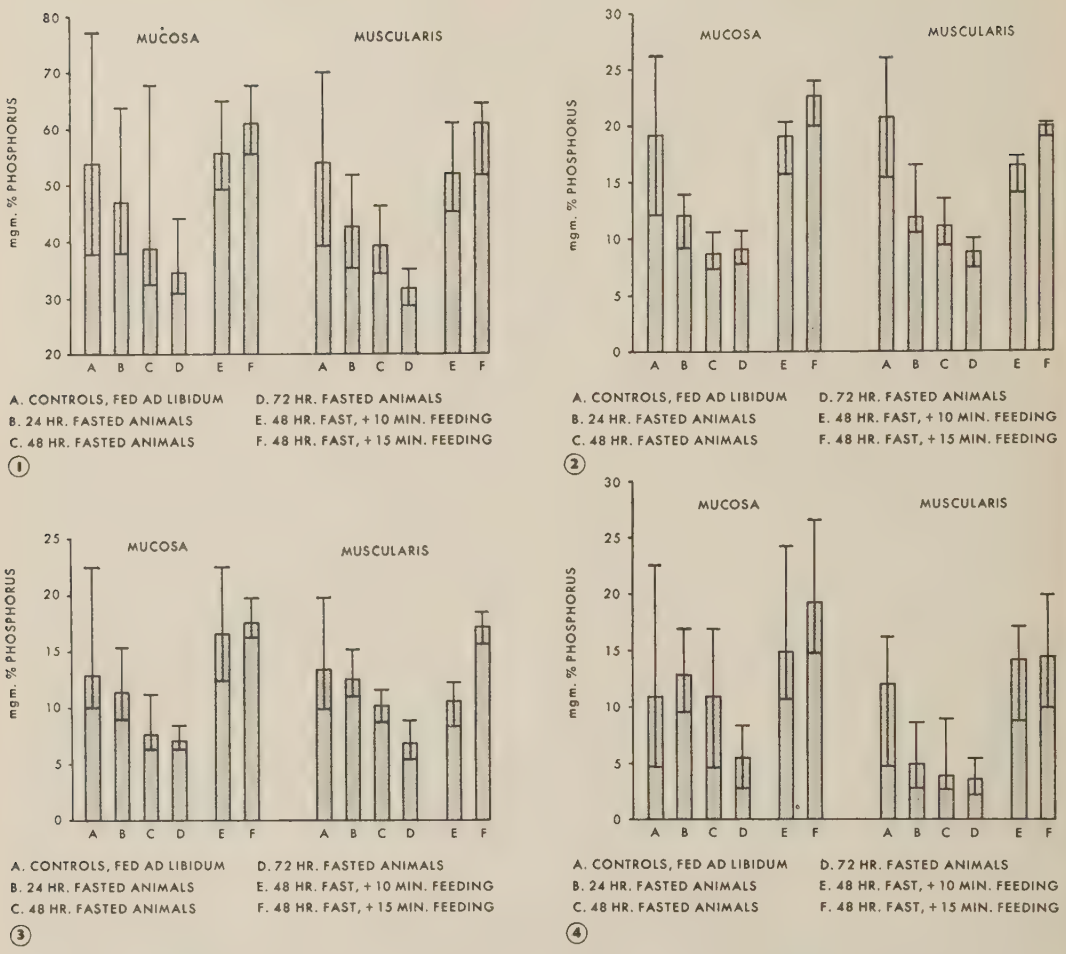

FIG. 1. Influence of fasting and feeding treatments on T.A.S.P.-P. concentrations in ileal segments of rat intestine. Concentrations shown as median values with quartile limits.

FIG. 2. Influence of fasting and feeding treatments on I.P.-P. concentrations in ileal segments of rat intestine. Concentrations shown as median values with quartile limits.

FIG. 3. Influence of fasting and feeding treatments on Cr.P.-P. concentrations in ileal segments of rat intestine. Concentrations shown as median values with quartile limits.

FIG. 4. Influence of fasting and feeding treatments on A.T.P.-P. concentrations in ileal segments of the rat intestine. Concentrations shown as median values with quartile limits.

tion. A.T.P.-P. values in the muscularis fall off rapidly during first 24 hours but decrease more gradually during next 2 days of fast.

When animals are allowed to nibble briefly for 10 and 15 minutes, there is a rapid return of all phosphate fractions towards normal and in many cases their values exceed controls. These altered values show the same uniformity as seen with fasted animals. Examination of alimentary tract showed no evidence of passage of food particles beyond the stomach and a few animals with marked responses had food particles only in their mouths.

Samples of tissue from all experimental groups were analyzed for both water and Kjeldahl nitrogen. These results indicated that changes in phosphate are not a concentration or dilution effect by water or other substances.

Discussion. Most values obtained in this study compare favorably with phosphate values reported for smooth muscle(6,7,8), however, the T.A.S.P.-P. content is considerably below those previously reported by Kroeger and Edwards(3). The finding that values for I.P.-P. and T.A.S.P.-P. decrease with fast-

ing are also in disagreement. No satisfactory explanation can be offered except those of strain difference and climatic conditions.

Adding the values for I.P.-P., Cr.P.-P. and A.T.P.-P. leaves rather small quantities of difficultly hydrolyzable phosphate. Values for this latter fraction do not show the decline exhibited by other fractions but remain quite constant. The trauma to which these tissues are exposed during separation is appreciable, although ortho phosphate content is not excessive.

Wide variation of concentration values for various fractions in the control group is perhaps best accounted for by animals' random eating patterns. Some animals may have just eaten, others not for several hours. As animals are subjected to more uniform treatment, range of concentration decreases. The apparent increase of A.T.P.-P. in the mucosa after 24-hour fast is perhaps accounted for by this mechanism, however this may be an example of transfer of energy-rich phosphate bonds from a storage unit to an active donor. With increased starvation time, even the donor becomes greatly depleted. The brief feeding of 48-hour fasted animals, causing shifts of phosphate values towards control amounts, suggests a mechanism responsible for preparing intestines for receiving and handling of food material. Incomplete data shows a similar picture for 24- and 72-hour fasted animals. Rapidity of these alterations indicates that amounts of energy-rich phosphate compounds are more dependent on activity states than purely nutritional depletion and restoration. A study of this "chemical reflex" may prove of value in determining whether hormonal and/or nervous mechanism is responsible for activation of intestinal tract. Investigation is now underway to determine sensory mechanisms for this reflex.

Summary. 1. Both muscularis and mucosa of rat show significant changes in amounts of energy-rich phosphate compounds when animal is fasted or fasted and briefly fed. 2. With increasing periods of starvation to 72 hours, the easily hydrolyzable phosphate compounds, Cr.P.-P. and A.T.P.P., diminish in both tissues in a predictable manner. 3. The mucosa appears to be more labile of the 2 tissues, although the muscularis shifts in the same direction and to nearly the same magnitude. 4. The assay of either Cr.P.-P. or A.T.P.P. on whole intestinal segments will provide sufficient data as would the independent assay of each, in the major tissues comprising the intestine, to follow the nature of the mechanism involved.

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Fluorometholone, A Preferentially Anti-Inflammatory Corticoid. (25049)

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Biological activities of hundreds of analogs and derivatives of hydrocortisone synthesized in the past decade have begun to establish certain patterns. Potencies far beyond that of hydrocortisone have become commonplace(1-5) and separation of "glucocorticoid" from "mineralocorticoid" properties has been achieved with several compounds. Many compounds which are potent in bioassays measuring changes in carbohydrate metabolism are also potent in bioassays measuring suppression of experimental inflammation, involution of lymphoid tissue, production of eosinopenia, and suppression of ACTH secretion. This

communication reports the biological profile of a new high-potency corticosteroid in which a marked divergence exists between "glucocorticoid" potency and anti-inflammatory potency in the rat. This compound is 6α-methyl-9α-fluoro-11β, 17α-dihydroxy-1,4-pregnadiene-3,20-dione, or fluorometholone(5,6).*

Methods. Fluorometholone was assayed in several test systems in comparison with appropriate standard compounds. In glycogen deposition assay(7) and granuloma pouch assay(8) it was compared with hydrocortisone, in ulcerogenic assay(9) it was compared with prednisolone, and in "mineralocorticoid" assay(7), the effect on excretion of electrolytes and on urine volume was compared with that of several other corticosteroids. All assays reported were performed with steroids given by subcutaneous route.

Results. Glycogen deposition assay. The calculated ratio of potencies (fluorometholone/hydrocortisone) in a typical assay was 24 (Table I). Seven such assays were performed and potency ratios were: 29, 21, 33, 40, 10, 24, 27. Mean potency ratio was 26.

Granuloma pouch assay. Table II shows results of 4 multiple-dose comparisons of fluorometholone with hydrocortisone. Potency ratios calculated from these data indicate the new compound to be 134, 123, 153, and 116 times as potent as hydrocortisone, for a mean ratio of 131.

Ulcerogenic assay. Experiments were performed to estimate ability of fluorometholone to produce ulcers in glandular portion of stomach of the fasting rat (Fig. 1). Prednisolone was the standard compound. Although quantitative relationships are not precise, inspection of the Figure permits an estimate that fluorometholone is about 15 $\times$ as potent as prednisolone in this respect. Since it was previously shown that prednisolone is 2 to 3 $\times$ as potent as hydrocortisone(9), it may be concluded that fluorometholone is 30 to 45 $\times$ hydrocortisone as an ulcerogenic agent.

Mineralocorticoid assay. Effect of graded doses of fluorometholone on urine volume and sodium output of adrenalectomized, salt-loaded rats was tested. Fluorometholone, at doses to 400 μg/rat, caused increased excretion of sodium and increased urine volume. Maximum sodium increase was 30% above control level at 100 μg/rat; increase was somewhat smaller when dose was increased to 400 μg. Maximum observed increase in urine volume was 100% above control, and was seen at 400 μg dose. This pattern is similar to those of prednisolone and 6α-methylprednisolone. It is, however, qualitatively different from that of 6α-methyl-9α-fluoroprednisolone (which differs from fluorometholone only by presence of 21-hydroxyl). The former compound produced *retention* of sodium at doses from 10 μg to 500 μg, reach-

TABLE I. Typical Glycogen Deposition Assay of Fluorometholone in Comparison with Hydrocortisone.

Compound	Dose* (mg/rat)	Liver glycogen (%)†
Hydrocortisone	.4	.58
	.8	.93
	1.6	1.78
Fluorometholone	.02	.88
	.04	1.21
	.08	1.56

* 5 rats/group. Steroids inj. subcut. as aqueous suspension. Single inj. 7 hr before sacrifice.

† Determined by anthrone method. Values are avg of 5 rats. Estimated potency ratio on this assay shows fluorometholone 24 $\times$ hydrocortisone.

* *Oxylone,* Registered Trade Mark, The Upjohn Co., Kalamazoo, Mich.

TABLE II. Granuloma Pouch Assays of Fluorometholone in Comparison with Hydrocortisone.

Compound	Dose* (mg/rat/day)	Mean vol of exudate in pouch (ml)† Exp. 1	Exp. 2	Exp. 3	Exp. 4
None		6.0	7.1	5.4	6.2
Hydrocortisone	.3				3.6
	.5	4.4	4.8	4.2	
	.6				2.2
	1.0	2.2	3.4	3.0	
	1.2				.3
	2.0	.4	.3	.6	
Fluorometho-	.0025				4.1
lone	.005		4.8	3.4	1.2
	.0075	2.2			
	.010		1.4	1.1	1.0
	.015	.3			
	.020		.4	.2	
	.030	0			

* No. of rats/group is usually 7. Range, 5-10. Subcut. injections as aqueous suspensions.

† Estimated potency ratios, fluorometholone/hydrocortisone: Exp. 1, 134; Exp. 2, 123; Exp. 3, 153; Exp. 4, 116. Avg of 4 ratios: 131.

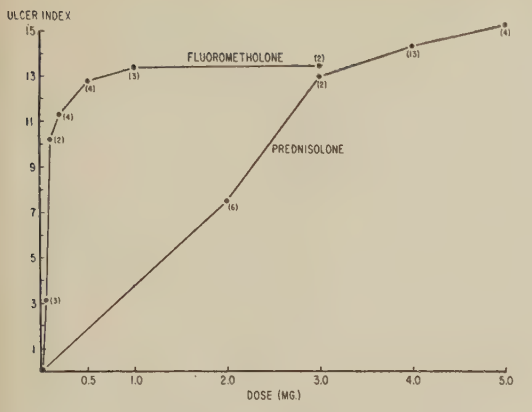

FIG. 1. Comparison of ulcerogenic potencies of fluorometholone and prednisolone. Numbers in parentheses refer to numbers of experiments performed for each dose, each experiment 10 animals. "Ulcer index" combines incidence, severity, and number of lesion/rat(9).

ing a maximal retention (65% of controls) at 200 μg; it caused some increase in urine volume, but only about 30% above controls at maximal dose, 200 μg.

Discussion. There are a number of remarkable things about the data presented. The most striking is the unusual relationship between anti-inflammatory and glucocorticoid potencies. In a large series of active corticoids, glucocorticoid usually exceeds or equals the anti-inflammatory potency. The situation here, *i.e.*, anti-inflammatory outstripping glucocorticoid potency by a ratio of 5:1, is one of the rare exceptions.

A second point of interest is the comparison between the electrolyte-regulating activities of fluorometholone and of 6α-methyl-9α-fluoro-prednisolone. The latter compound exhibits definite sodium-retaining properties (10), while fluorometholone, differing only by absence of 21-hydroxy group, has an opposite effect.

Potency of fluorometholone in producing gastric ulcers is about 30-45 times that of hydrocortisone. This ratio lies between the potency ratio of these 2 compounds in glycogen deposition and anti-inflammatory assays, although somewhat closer to the former. Thus, ulcerogenic potency of corticoids does not necessarily correlate with either of the other major activities.

The disparity between these potencies and those observed in other species by the systemic route is most interesting. Fluorometholone is less potent than prednisolone in producing eosinopenia and hyperglycemia in *man*(12), when given systemically. The fact that when fluorometholone is given *topically* to man, it has anti-inflammatory potency 40 × that of hydrocortisone(13) indicates that *at the effector site* the compound manifests high potency, but that whether or not it can retain its high activity while passing through the system depends on the species studied. It is possible that man has more efficient systemic mechanisms for destruction of fluorometholone than has the rat.

Summary. Fluorometholone is an unusual synthetic corticoid in the rat because its anti-inflammatory potency (131 × hydrocortisone) is much greater than its glucocorticoid potency (26 × hydrocortisone). Its potency in promoting development of ulcers of the pyloric portion of rat stomach is between its glucocorticoid potency and its anti-inflammatory potency. It does not cause sodium retention at doses in which a similar compound (differing only by presence of C-21 hydroxyl) does produce measurable sodium retention.

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Effect of Insulin, Fasting and Tolbutamide on Dextran Edema in Rats.* (25050)

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Insulin has been shown by Adamkiewicz to increase the rat's sensitivity to dextran(1). As a rule, dextran causes no reaction when injected subcutaneously, except after several hours(2), but when given by this route conjointly with insulin, it produces a prompt characteristic edematous reaction in paws, ears and snout. While confirming these observations, Goth further reported that insulin promotes histamine release induced by dextran(3). However, the rather high dosages of insulin used make it difficult to interpret the mechanism of this sensitizing phenomenon. The present communication describes experiments in which insulin was given in decreasing doses to rats treated with dextran; changes in blood sugar were recorded. The role of hypoglycemia as possible contributing factor to dextran susceptibility was also investigated in intact and adrenalectomized fasted rats and following treatment with tolbutamide.

Methods. One hundred and sixty-eight female rats (Holtzman) weighing 100 to 120 g were used in 4 experiments. They were kept on Purina Fox Chow and received tap water *ad lib.* Whenever food was withheld, animals had access to water. Bilateral adrenalectomies were performed 24 hours before start of experiments and rats received, as maintenance therapy, a single subcutaneous injection of 1 mg of desoxycorticosterone acetate in the form of aqueous suspension of its microcrystals. Dextran, a commercial 6% solution (Abbott), was administered at dose of 0.3 ml, subcutaneously in scapular region. Zinc-insulin (Connaught) was also injected at same time, subcutaneously, in the inguinal region. Tolbutamide (Horner) was given by stomach tube at dose of 30 mg in 0.5 ml of physiologic saline, 30 minutes prior to injection of dextran. Degree of edema of paws and snout was appraised according to arbitrary scale of + to ++++. Readings were made 1 hour after dextran injection and scores were alloted to each degree so that + (traces) = 1; ++ = 2; +++ = 3; and ++++ = 4. Our results are expressed as percentages of maximal possible effect (++++). Values shown in Tables represent means of degrees of edema when present. Blood samples were withdrawn one hour and 15 minutes after dextran injection. They were taken with heparinized syringe from jugular vein, under ether anaesthesia; sugar determinations were made according to method of Folin-Wu.

Results. Table I lists results of 2 experiments in which rats received increasing doses of insulin. Both experiments were identical, except that in the second, animals were fasted for 12 hours before receiving insulin and dextran. In both cases, there was a maximal sensitizing effect to dextran when 0.5 unit of insulin was used. Higher doses did not increase the intensity of edema reaction; on the contrary, there was a decrease in response proportional to lowering of blood sugar. This is more evident in Exp. 2, where the 12-hour fast so increased the hypoglycemic effect of insulin that all animals receiving 2 units were dead after 4 hours. However, one fasted rat not treated with insulin showed a slight increase in sensitivity to dextran.

The third experiment was devised to evaluate the influence of fasting alone on dextran edema. Twelve to 48 hours of fasting did not promote sensitivity to dextran in intact animals and blood sugar levels remained unaffected (Table II). This situation was totally

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† Medical Research Associate of Nat. Research Council of Canada.

TABLE I. Effects of Insulin on Edema Reaction to Dextran Injected Subcutaneously in Rats.

Insulin (I.U.)	Exp. I: No fasting (10 rats/group) Edema* Incidence	Edema* Degree (%)‡	Glycemia, mg %	Exp. II: 12 hr fasting (8 rats/group) Edema* Incidence	Edema* Degree (%)‡	Glycemia, mg %
.0	0			1	25	98 ± 3.4
.1	8	25		8	32	67 ± 4.4
.25	8	50		8	38	63 ± 3.6
.5	10	72	120 ± 5.7	7	56	45 ± 3.9
1.	10	65	89 ± 3.9	7	50	46 ± 3.4
2.	9	56	57 ± 4.7	8	38	39† ± 1.6

* Mean readings 1 hr after inj. of dextran (0.3 ml 6% s.c.).
† All rats died within 4 hr after insulin inj.
‡ Mean percentage of degrees of edema when present.

reversed in adrenalectomized animals; prolonged fasting was particularly efficient in increasing susceptibility of rats to dextran. The 48-hour fasted adrenalectomized rats remained edematous as long as they were kept without food even though they had received only a single injection of dextran; this is in contrast with usual fleeting appearance of dextran edema, which usually lasts no more than a few hours.

Table III summarizes our observations on the influence of tolbutamide upon dextran reaction. Hypoglycemic sulfonamide had a more potent sensitizing action on dextran edema than insulin. This effect was produced without much alteration of blood sugar level. Here, the fasting period was purposely restricted to 6 hours before administration of tolbutamide, followed half an hour later by dextran injection.

TABLE II. Effect of Fasting on Edema Reaction to Dextran Injected Subcutaneously in Intact and Adrenalectomized Rats.*

Treatment	Fasting period, hr	Edema† Incidence	Edema† Degree, %‡	Glycemia, mg %
	12	0		110 ± 5.4
	48	0		108 ± 6.1
Adrenalectomy + DCA	12	2	25	85 ± 4.3
Idem	48	10	100	66 ± 4.2

* 4 groups, 10 rats each.
† Mean readings 1 hr after inj. of dextran (0.3 ml 6% s.c.).
‡ Mean percentage of degrees of edema when present.

Discussion. Our study reveals that the influence of insulin on dextran reaction in rats is not necessarily related to hypoglycemia. Furthermore, sensitivity to dextran may be augmented by fasting in adrenalectomized rats. Whether the latter condition is attributable to a higher level of endogenous insulin or merely the result of adrenal insufficiency remains to be demonstrated. The action of tolbutamide on dextran edema is probably mediated by insulin(4); it occurs without significant change in blood sugar values. Should variations in glycemia be involved in this process of sensitization, they would fall within the physiologic range. In this regard, let us recall that, according to Edlund, the inhibitory effect of alloxan upon dextran edema is also demonstrable in absence of blood sugar changes(5).

It is still controversial whether the increase in capillary permeability produced by dextran is attributable to direct vasotoxic action or secondary to histamine release(6). Goth recently reported that insulin augments release of histamine produced by dextran, but not that produced by 48/80(3). This accords with our own experiments (unpublished) in which insulin exerted no promoting action on

TABLE III. Effect of Tolbutamide on Edema Reaction to Dextran Injected Subcutaneously in Rats.*

Treatment	Fasting period, hr	Edema† Incidence	Edema† Degree, %‡	Glycemia, mg %
	6	0		125 ± 5.3
Tolbutamide (30 mg orally)	6	10	97	97 ± 3.9

* 2 groups, 10 rats each.
† Mean readings 1 hr after inj. of dextran (0.3 ml 6% s.c.).
‡ Mean percentage of degrees of edema when present.

production of edema in rats treated with 48/80. However, the increase of histamine liberation by dextran with insulin may be secondary to augmentation of edema reaction *per se*.

Goth's hypothesis that insulin may promote entry of certain large molecules of a histamine releasing agent(3) is perhaps the most valid one. It would be in accord with known effect of insulin on cell membrane permeability for glucose. Besides, the influence of insulin on dextran edema consists more in precipitating than in augmenting the reaction. Consequently, the sensitizing action of insulin may be explained either by increased rate of penetration of dextran into cells capable of liberating histamine or through accelerated systemic absorption of dextran with resulting toxic effects.

Summary. Insulin in doses that are not hypoglycemic increases the rat's sensitivity to dextran reaction, characterized by edema. A comparable effect was observed in adrenalectomized fasted rats and following treatment with tolbutamide.

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Absence of Gene Interaction in Mouse Hybrids, Revealed by Studies of Immunological Tolerance and Homotransplantation.* (25051)

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It has recently been suggested(1,2) that the well-established phenomenon of immunological tolerance and homotransplantation immunity be used to ascertain whether or not genic interaction in antigen production occurs in inbred strains of mice. Both Haldane and Fox suggested that the hypothesis of genic interaction in mice might be tested as follows. Animals of one inbred strain of mice may be made tolerant of another inbred strain by injection of hematopoietic cells at birth, then skin from F_1 hybrids grafted to tolerant representatives of parent strain. In addition, animals of one parent strain made tolerant by injection of hybrid cells at birth could be tested by homotransplantation with skin from homologous parent strain. Under these conditions, if the tolerant parent rejects the F_1 transplant, this might be taken as convincing evidence that the F_1 hybrid possesses histocompatibility antigens not present in either of parent strains. If, however, the graft is accepted by the tolerant parent, this would be evidence that the F_1 individual carries all antigens present in both parent strains and would lend support to the one-gene-one-antigen theory previously formulated by Haldane. The observations here reported appear to be compatible with the latter hypothesis.

Method. Highly inbred mice of A and C3H strains and their reciprocal F_1 hybrids were used. In the first experiments, tolerance of A tissue in C3H mice was induced by injecting C3H animals at birth with approximately 4 million viable spleen cells taken from an adult A donor. The technic for spleen cell injection has been previously described(3). Approximately one month following weaning, treated mice were submitted to skin grafts taken from adult donors of either A strain or (A x C3H) F_1 animals by the method previously described(3). Both donor and recipient animals were always of the same sex and approximately of same age. Grafts were made when recipient mice were

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TABLE I. Immunological Tolerance of Parent Strain and Hybrid Skin Homotransplants.

Recipient strain	Spleen cells inj. at birth†	No. of mice	Successful skin homografts* A skin	Successful skin homografts* (A × C3H)F$_1$ skin
C3H	A	15	12/15	7/7
	A	9		7/9
	(A × C3H)F$_1$	26	16/26	
	"	10		6/10
	None	24	0/12	0/12

* Numerator indicates No. of skin grafts successful; denominator, No. of grafts attempted.
† 4 million viable spleen cells inj. intrav. in anterior facial vein during first 24 hr of life.

approximately 2 months of age and the success of the transplant was judged by persistence of a healthy skin graft for at least 2 months. In evaluating success of the graft, the direction of hair growth was of paramount importance, since the method of grafting involves a 180° rotation of donor skin prior to implantation on the recipient. Further, successful grafts were regularly noted to grow along with growth of host. In second set of experiments, C3H mice were made tolerant of the (A x C3H) F_1 by injection of approximately 4 million spleen cells from adult hybrids at birth. Two months later homotransplantation of skin taken from either (A x C3H) F_1 or A donors was attempted. Here again mice were followed for at least 2 months after grafting and success or failure of the graft determined, as described in the first experiment.

Results. The results are summarized in Table I. Of 15 mice of the C3H strain treated at birth with A spleen cells, 12 (80%) were tolerant and accepted skin grafts from homologous A strain mice. In 7 of these animals a second skin transplant taken from (A x C3H) F_1 hybrids was also successful. Of another group of 9 C3H animals treated at birth with A spleen cells, 7 (78%) were tolerant of the (A x C3H) F_1 skin. Similarly, when tolerance was induced in C3H animals by injection of (A x C3H) F_1 spleen cells, 16 out of 26 (61%) accepted the A skin, and 6 out of 10 of another group of C3H animals similarly treated accepted the (A x C3H) F_1 skin. Nontreated C3H controls grafted with either A or (A x C3H) F_1 skin always rejected these homografts.

Fig. 1 illustrates a C3H mouse, pretreated at birth with A spleen cells, bearing both A and (A x C3H) F_1 skin grafts.

Discussion. The results show that when tolerance in one inbred strain of mice is in-

FIG. 1. C_3H mouse pretreated at birth with A spleen cells bearing both A (below broken line) and (A × C_3H) F_1 (above broken line) skin grafts.

duced by injecting at birth spleen cells from individuals of another inbred strain, the recipients accept skin grafts taken from mice of the strain donating the spleen cells as well as skin taken from F_1 hybrids resulting from the cross of the 2 strains involved. Similarly, when tolerance is induced in one of the parents by injection of spleen cells from F_1 hybrid, the pretreated mice accept not only skin grafts from the hybrid but also skin grafts from the other parent strain.

These observations indicate, first of all, that individuals of the F_1 hybrid of the 2 strains involved share all the histo-compatibility antigens present in each parent strain. Perhaps of greater significance are the experiments demonstrating that no new histo-compatibility antigens appear as a consequence of hybridization. These observations do not exclude completely the possibility that in mice, as in other species(4,5), new antigens not present in either parent strain may appear as a consequence of genic interaction. However, the investigations do demonstrate that, if such be the case, transplantation studies and employment of systems including immunological tolerance are inadequate for detection of such antigens and the responsible genic interaction. In spite of these results, it seems possible that other techniques, i.e., immunochemical methods or even study of the phenomenon of runt diseases, may be more fruitful in detecting evidence of the postulated genic interaction.

Summary and conclusions. 1. C3H mice made tolerant by injection of A spleen cells at birth are tolerant of skin homografts from A strain and (A x C3H) F_1 donors. 2. C3H mice made tolerant by injection of (A x C3H) F_1 spleen cells at birth are tolerant of skin homotransplants from both A strain and (A x C3H) F_1 donors. These observations are interpreted as evidence indicating that new histocompatibility antigens do not derive from genic interaction during hybridization in the 2 inbred strains of mice in these studies.

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Separation of Thrombin from Thrombokinase by Continuous Flow Paper Electrophoresis.* (25052)

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For several years, thrombokinase has been prepared in this laboratory from bovine plasma. It is heat-labile and associated with globulins. However, its identifying characteristic is its capacity to activate prothrombin. In this function it does not act like platelets, but rather is complemented by platelets(1). Recently, thrombokinase has been purified by a method which yields about 1.2 mg/liter of plasma(2). This material has now been subjected to continuous flow paper electrophoresis.

Methods and materials. Electrophoresis was performed in Spinco Model CP cell enclosed in refrigerator at 1.3 to 2.9°C. Buffer: veronal, pH 8.6, ionic strength, 0.02. Current: 50 ma; 807 volts. 77 ml thrombokinase, representing 77 liters of plasma, was diluted with 1463 ml of 0.02 M acetate, pH 5.2. The precipitate was dissolved in veronal buffer to make 25.7 ml of solution, which had the same

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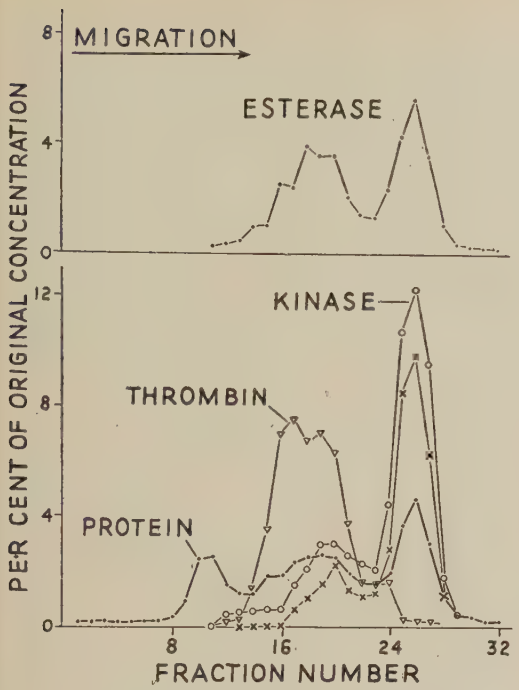

FIG. 1. Continuous flow paper electrophoresis of thrombokinase prepared from bovine plasma. There are 2 curves for kinase activity. The upper one presents assays performed in presence of oxalate; the lower one gives values obtained in 5-reagent system.

conductivity as the buffer. This "original solution" was applied at rate of 0.43 ml/hour to the extreme left tab of paper curtain. Thrombokinase was assayed by its capacity to activate prothrombin in presence of oxalate, and also in a 5-reagent system containing prothrombin, cephalin, calcium, bovine barium carbonate serum and sample to be tested(2). In each case relative activity was derived from a reference curve obtained with dilutions of original solution. Thrombin was estimated as described(2), with dry sample of NIH thrombin as the ultimate standard. Esterase activity on tosylarginine methyl ester (TAMe) was determined by method of Sherry and Troll(3). Protein was estimated by method of Lowry *et al.*(4) with crystallized bovine albumin as standard; and the values are subject to limitations noted by them.

Results. A large proportion of thrombokinase was separated in the rapidly moving peak shown in Fig. 1. In fraction 26 the kinase activity was 12.3% of the original, and the protein was 4.6%. Hence specific activity was 2.7 times that of the original solution.

A trail, or second smaller rise of kinase activity occurred in all 5 such analyses. For one of these, the kinase had been dialyzed overnight against the veronal buffer. The trailing effect might be due to association of proteins as complexes in the middle protein band. It is not necessary to assume that 2 different kinases were separated; although that possibility must be considered. The fact that the 2 measures of kinase activity gave essentially the same result for the peak and the trailing hump, indicates that the same factor could be responsible for the activity in both regions. The difference between the 2 kinase curves is within limits of error. For assay of kinase in presence of oxalate, fraction 26 was diluted 1/40, to protein concentration of 5.3 μg/ml. For assay of kinase in the 5-reagent system, fraction 26 was diluted 1/100,000 to a concentration of 0.0021 μg/ml.

The original solution had a high kinase and low thrombin activity. It contained only 68.3 thrombin units/ml or 14.8 units/mg protein. The moderately high thrombin peak in Fig. 1 represents a high percentage yield rather than a high activity.

The original solution had 966 esterase units/ml or 209 units/mg protein. The Figure suggests that esterase activity is associated with both thrombin and kinase. Much of esterase activity migrated with kinase in the leading peak where there was very little thrombin. In terms of percentage scale of Fig. 1, the ratio of esterase to kinase was 0.40, 0.46 and 0.37 for fractions 25, 26 and 27, respectively. Fraction 26 had 251 esterase units/mg.

Discussion. Gross separation of thrombin from thrombokinase was achieved by continuous flow electrophoresis. But such separation had been accomplished previously by repeated fractionation with ammonium sulfate(1); and it is not certain that electrophoresis is superior as a method of preparation.

From the analytic viewpoint, the electrophoretic procedure offered several opportunities. One of these was comparison of values obtained when kinase was assayed with and

without complementing reagents. It appears that thrombokinase can activate prothrombin, unaided by other factors(5). But thrombin is produced much faster if the system is complemented by platelets or cephalin, provided that ionic calcium is also added. Production of thrombin is further accelerated by the serum reagent. Together, these reagents enormously magnify the effect of kinase. When minute amounts of kinase are assayed in this system, serious uncertainties are involved(2). Therefore, it is of interest that assays in this complex system paralleled those with oxalated prothrombin; although the former system was 2500 times as sensitive.

In some respects, thrombokinase resembles trypsin, which also can activate prothrombin in presence of oxalate. TAMe is a good substrate for trypsin(6); and others have anticipated the possibility that TAMe might be a substrate for natural activator of prothrombin (7). The present results suggest that TAMe may well be a substrate for thrombokinase; but more detailed study of this point is desirable.

Summary. Thrombokinase prepared from bovine plasma was further purified by continuous flow paper electrophoresis. Thrombokinase was assayed by its capacity to activate prothrombin in the presence of oxalate, and also by production of thrombin in a system containing prothrombin, cephalin, calcium and bovine "barium carbonate serum." Curves of these 2 assays were similar and formed a peak ahead of the thrombin peak. TAMe esterase activity appeared in 2 peaks which corresponded respectively to thrombin and thrombokinase peaks.

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Genetics of Human Cell Lines I. 8-Azaguanine Resistance, a Selective "Single-Step" Marker.* (25053)

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From the methodological point of view, the mammalian cell grown *in vitro* can be regarded as a unicellular microorganism. Thus, methods developed for quantitative work with microbes can be adapted to the genetic study of human cells, provided suitable selective markers are available. Plating and colony-counting technic, so useful for assay of viable microbial cells, was introduced into tissue culture methodology by Puck *et al.*(1). Our purpose was to find a suitable mutational system for mammalian cells in which, under selective conditions, mutant cells survive and form well developed colonies while parental population is completely inhibited or eliminated. Mutation from 8-azaguanine (AG) sensitivity to resistance satisfied the foregoing criteria(2). Moreover, the property of 8-azaguanine resistance appears to be an excellent genetic marker, since it is not associated with modifications in morphological or cultural characteristics of cells either in presence or absence of the selective agent.

Materials and methods. Strain *Detroit-98* (D98), derived from human sternal marrow by Berman and Stulberg(3), was kindly supplied by Dr. H. Moser of Cold Spring Harbor Labs. A single-cell-derived clone D98S was used throughout these studies. *Media.* The basic medium was essentially that described by Eagle(4), containing 10% com-

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plete horse serum. Sterilization was effected by filtration through Selas No. 2 filter. *Cultivation and plating procedures.* The methods were based on those developed by Puck *et al.*(1) and adapted for strain D98 by Moser and Tomizawa(5). Cells were grown on glass surface of 60-mm Petri dishes at 37°C in 5-10% CO_2 atmosphere. The inoculum was prepared from a 3- to 6-day-old culture of vigorously growing cells, washed serum-free with balanced salt solution before detachment and dispersion in the same solution containing 0.25% pancreatin (Nutritional Biochemical Corp., Cleveland) (5-minute incubation at 37°C followed by gentle manual shaking). The action of pancreatin was terminated by addition of serum-containing medium, after which the cell suspension was filtered aseptically through fine cheesecloth to remove any cell clumps. The cell density of this essentially monodispersed suspension was assayed in a hemacytometer. For colony counts, cells appropriately diluted in 5 ml of serum-containing medium were plated and incubated 6 to 12 days, with a complete medium change every 2 to 3 days. Growth was assayed either by counting and measuring colonies or by protein determination. *Protein determination* is based in part on protein staining method of Durrum(6) and its modifications for electrophoretic(7) and cytochemical(8) purposes. Adaptation of these methods for quantitative protein determination in mammalian cell cultures, suggested by Dr. A. W. Kozinski of this laboratory, permits determination of total protein without disturbing colonies on the glass. Thus a colony count may be performed on the same plate after completion of protein determination. The assay is based on spectrophotometric determination of bromphenol blue selectively bound by the protein component of $HgCl_2$-fixed cells and subsequently eluted with alkaline acetone. The procedure consists of: (1) thorough washing of glass-attached cells with balanced salt solution to remove all serum proteins; (2) 15-minute staining at 37°C with aqueous solution containing 0.1% bromphenol blue and 5% $HgCl_2$; (3) three consecutive washes with 0.5% acetic acid each lasting 6 minutes; (4) quantitative elution with 3-ml portions of aqueous acetone (20 ml acetone 0.2 ml 10 N NaOH, 5 ml water, freshly prepared); (5) adjusting the collected extracts to 10-ml volume and measuring optical density at 595 mμ wave length against a blank prepared similarly from cell-free plate preincubated with serum-containing medium. The actual amount of cell protein/plate is ascertained from predetermined calibration curves. The same procedure was also used for cell suspensions, with centrifugation as an added step. *Colony count.* After fixation in Bouin's fluid or after protein determination, colonies were stained with Giemsa solution. The dry dishes were then inserted into a projector (Bausch & Lomb, "Tri-Simplex" micro-projector), and well-focused images of colonies were scored by an electronic counter (built by Philip D. Mintz of this laboratory).

Results. When above procedures are followed, strain D98S exhibits a consistent plating efficiency of greater than 0.6, *i.e.*, over 60% of microscopically assayed cells give rise to well defined colonies (Fig. 1), which can easily be counted, providing their number does not exceed 5000/plate. Above this figure, colonies coalesce, and total protein synthesis after 14 days' incubation becomes less than proportional to the number of cells in the inoculum (Fig. 2).

A series of plates was prepared, each seeded with approximately 5000 cells, to which AG was added in graded concentrations. Fig. 3 represents the survival curve 1 of the wild-

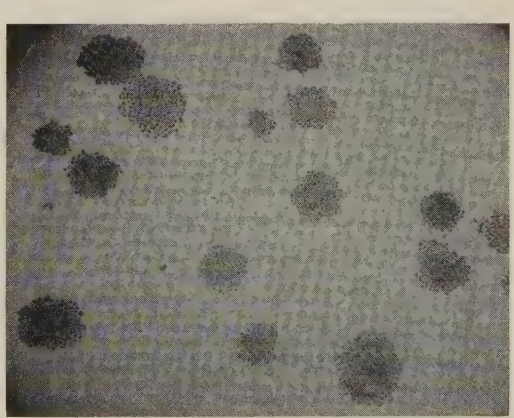

FIG. 1. 6-day-old colonies of strain D98S grown on glass, Bouin fixed, Giemsa stained.

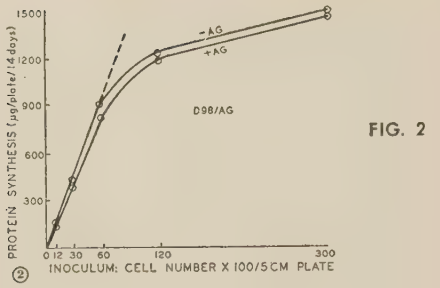

FIG. 2

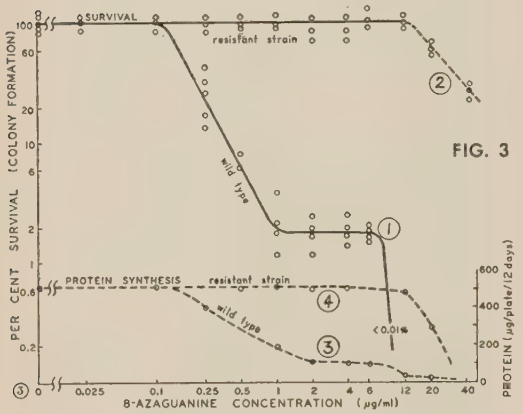

FIG. 3

FIG. 2. Relationship between inoculum size (colony-forming cells) and total growth determined as amount of protein synthesized (14 days) by AG-resistant strain D98/AG grown in presence (+AG) or absence (–AG) of 8 μg AG/ml medium.

FIG. 3. 12-day growth determined as amount of protein synthesized and survival (colony formation) of AG-sensitive (wild type—1, 3) and AG-resistant (2, 4) isolates derived from strain D98S grown at increasing concentrations of AG. Inoculum was 5000 cells/plate.

type strain D98S. It is apparent that in presence up to 0.1 μg AG/ml survival and colony-forming ability of each cell is virtually unaffected. There is a sharp drop in plating efficiency between 0.1 and 1 μg AG/ml, followed by a plateau extending to approximately 10 μg AG/ml, at which level 1 to 2% of cells survive and form colonies. These colonies were isolated and found to be stable, AG-resistant clones (D98/AG) as reflected by survival curve 2. Their growth, as measured by protein synthesis, was not affected by AG in concentrations up to 12 μg/ml (curve 4), while protein synthesis of the wild-type strain was increasingly suppressed above 0.1 μg/ml.

Thus the wild-type population contains approximately 1 to 2% mutant cells, exhibiting to 100-fold increase in AG resistance without impairment of plating efficiency or growth rate either in the presence or in the absence of AG (Fig. 2 and 3). Fig. 4 shows appearance of colonies on 2 plates seeded with equal inoculum of AG-sensitive strain D98S, incubated in absence (A) and in presence (B) of AG (6 μg/ml).

Resistance to AG appears to be a highly stable property. Subculture for over 150 generations, either in absence (curve 3) or in presence (curve 2) of AG (6 μg/ml) did not alter level of resistance (Fig. 5). By subculturing resistant lines at high concentrations of AG, strains with slightly higher resistance could be isolated (Fig. 5, curve 4).

Determination of mutation rates was based on Luria-Delbrück variance test(9), modified for mammalian cells grown on glass. Each colony grown in absence of AG and firmly attached to glass may be considered as analogous to one "test tube culture"(9). On addition of the drug (AG), only colonies containing at least one resistant cell would give rise to secondary resistant colonies, while others would slough off the glass and be lost during medium changes. Mutation rate based on preliminary experiments, employing the P_0 method(9) corresponds to 5 x 10^{-4}/cell/generation.

Properties of AG-resistant cells. AG-sensitive cells were found to exhibit the same inhibitory threshold for AG and 8-azaguanosine (10^{-7} M). Neither guanine (1 to 50 μg/ml) nor adenine nor thymine antagonize the inhibitory action of 1 to 50 μg/AG/ml. AG-resistant lines are approximately 100 times more resistant to AG, but only 2-3 times more resistant to 8-azaguanosine.† They were also found to be more resistant to 5-fluorouracil and to ultraviolet (UV) light (Fig. 6 and Fig. 7, curve 3). It was therefore of interest to

† Strain D98/AG contains approximately 0.01% mutants (D98/AGR) resistant in a single (genetic) step to over a 100-fold higher concentration of 8-azaguanosine (AGR), with concomitant further increase in AG resistance (over 10-fold). Direct mutations from AG sensitivity (D98S) to AGR resistance (D98/AGR) were not detected in populations as large as 10^7 cells. Thus AG and AGR resistances behave as two sequential mutational steps.

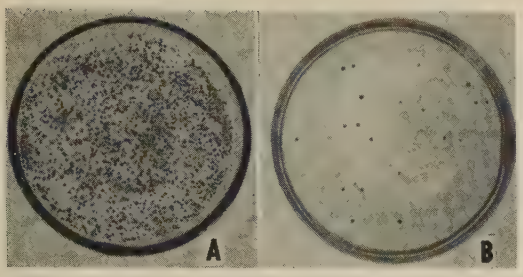

FIG. 4. Colony formation on 2 plates seeded with identical inocula (4000 cells) of AG-sensitive cells (strain D98S) in absence (A) or presence (B) of 6 μg AG/ml medium.

determine whether UV would increase mutation frequency from AG sensitivity to resistance or would select for the more UV-resistant, AG-resistant cells(10). The wild-type population was irradiated with increasing doses of UV and plated in absence (curve 1) or in the presence (curves 2 and 2a) of 6 μg AG/ml. It is apparent that AG-resistant cells not exposed to AG prior to irradiation, exhibit a "single-hit" survival curve (curves 2 and 2a), while UV survivals of parental strain D98S (curve 1) and of the established resistant line D98/AG (curve 3) are characterized by "multi-hit" curves. Curve 4 represents UV survival of the AG-resistant line at slightly inhibitory concentrations of AG (12 μg/ml). The shape of survival curve appears unchanged. It may therefore be concluded that the newly emerging AG-resistant cells, pre-existing in the wild-type population, contain only one AG-resistant "complement" (chromosome?), while the number of these "complements" increases during establishment of the resistant sublines. Further genetic and cytological studies may explain this phenomenon and indicate whether processes analogous to nondisjunction or mitotic crossing-over could be involved.

Summary. A cloned wild-type population (strain D98S) derived from human bone marrow cells, which is inhibited by 8-azaguanine (AG) in concentrations in excess of 0.1 μg/ml, contains approximately 1 to 2% cells resistant to AG in 100-fold higher concentrations. A single-step mutational process (in broad meaning of the word) appears to be involved. The AG-resistant lines are stable upon prolonged subculture either in presence or absence of drug. Mutation rate from sensitivity to resistance is of the order of 5 x 10^{-4}/cell/generation. AG resistance is a use-

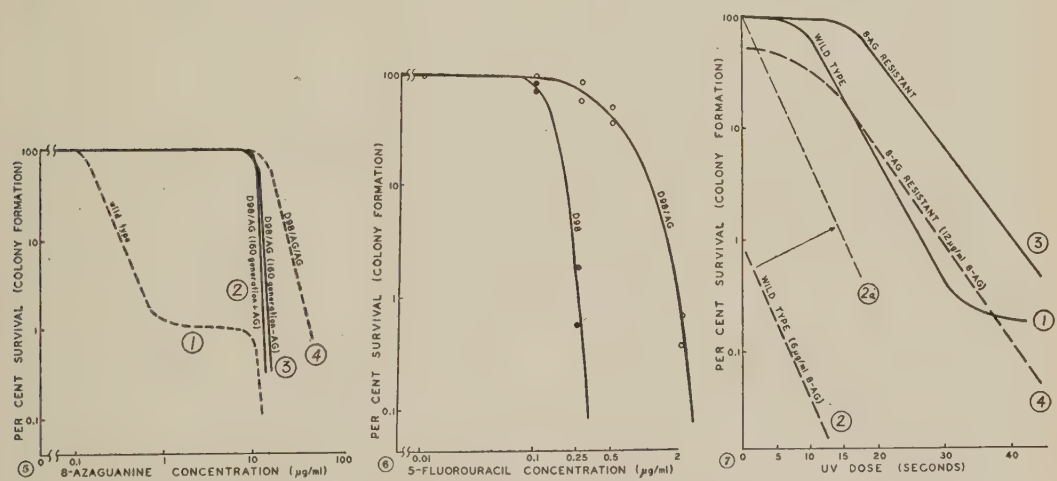

FIG. 5. Survival of AG-sensitive (1) and of AG-resistant mutant lines at increasing concentrations of AG. Curves 2 and 3 represent survival of AG-resistant strains after subculture for 160 generations in presence (2) or absence (3) of 5 μg AG/medium. Survival of more resistant line D98/AG/AG, obtained by serial subculture of D98/AG at 20 to 40 μg AG/ml, is depicted by curve 4.

FIG. 6. Survival of AG-sensitive (D98) and AG-resistant (D98/AG) strains at increasing concentrations of 5-fluorouracil.

FIG. 7. Ultraviolet survival of AG-sensitive (1, 2) and AG-resistant (3, 4) cell lines grown in absence (1, 3) or presense (2, 4) of AG. Curve 2a is parallel to curve 2. UV intensity 120 μW/cm^2.

ful genetic marker, since at selective AG concentrations (a wide plateau at 1-10 μg/ml) colony formation by resistant cells is not impaired and sensitive cells are destroyed. A method for protein determination is described, based on measurement of eluted bromphenol blue from $HgCl_2$-fixed, glass-attached cells.

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Mechanism of Inhibition of Gastric Secretion in the Rat Following Bile Duct Ligation.* (25054)

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Madden *et al.*(1) noted that simultaneous bile duct ligation in the Shay rat preparation prevented formation of rumenal ulcers. Ambrus(2) found that gastric secretory output from gastric fistula rats was slightly decreased when the bile duct was ligated. To date, this interesting phenomenon has not been adequately explained. On the basis of some evidence(3) that bile, when present in the intestine, is a gastric secretagogue, it was thought that removal of bile from the intestine could lead to decrease in gastric secretory activity. Actually, it has been postulated on the basis of a possible secretagogue effect of bile that continuous entrance of bile into the intestine in the rat, by virtue of absence of gall bladder, could explain the large interdigestive gastric secretion so characteristic of the rat(4). Our data show that gastric secretion in the rat is inhibited by bile duct ligation. This inhibition is due, not to removal of bile from the intestine, but to inhibitory effects of bile salts on gastric secretion.

Materials and methods. Gastric secretion was measured by pylorus ligation method(5) in 151 male Holtzman rats weighing from 160 to 220 g. Prior to experiments, the rats were fasted for 48 hours in individual cages with wide mesh wire bottoms to prevent coprophagia. Water was given *ad lib.* during fasting period. Following the 48 hour fast, the rats were operated under light ether anesthesia and under clean conditions and the pylorus gently ligated with a 4-0 silk ligature. Simultaneous ancillary procedures were done and will be described in relation to specific test groups. Exactly 4 hours later (6 hours in one group) the animals were killed by exsanguination, the stomachs removed and gastric content studied individually for volume and free acidity. The rats were divided into 5 groups.

Results. Group 1. Simultaneously with pyloric ligation, the proximal portion of bile duct was divided between 2 ligatures. In random controls, the duct was merely exposed. There was inhibition in volume and free HCl concentration of gastric content in bile duct ligated rats (Table I). There was relatively greater inhibition in volume than in free HCl concentration.

Group 2. An external biliary fistula was created simultaneously with pyloric ligation. This was accomplished by catheterizing proximal portion of bile duct with a fine polyethyl-

* The authors wish to acknowledge the valuable technical assistance of Mr. Howard Guiles.

† Markle Scholar in Medical Sciences.

TABLE I. Effect of Bile Duct Ligation, External Biliary Drainage, Internal Diversion of Bile from Small Intestine and Parenteral Administration of Bile Salts on Gastric Secretion in Pylorus Ligated Rats.

Group		No. of rats	Wt (g)*	Vol (ml)*	Free HCl (meq/l)*
1	Controls	12	208 ± 12.68	7.20 ± 1.90	112 ± 13.07
	Bile duct ligation	14	209 ± 16.55	1.25 ± .68 p <.001	62.5 ± 19.0 p <.001
2	Controls	10	204 ± 9.64	2.63 ± .96 p <.001	83 ± 13.6 p <.001
	Ext. biliary fistula	13	213 ± 8.60	3.46 ± 1.47 p <.001	104 ± 15.45 p <.001
3†	Controls	12	165 ± 10.48	9.80 ± 1.90 p <.001	107 ± 9.38 p <.001
	Bile shunt	10	160 ± 10.77	9.14 ± 1.60 p <.001	106 ± 10.0 p <.001
4	Controls	12	220 ± 23.70	7.44 ± 1.50	109 ± 12.80
	20% sodium dehydrocholate, 150 mg/200 g by I.V. infusion	12	224 ± 28.10	.65 ± .90 p <.001	36 ± 41.94 p <.001
5	Controls	19	182 ± 18.30	6.59 ± 1.59	110 ± 10.44
	Ox bile salts, 20 mg/200 g intraper. inj.	18	174 ± 16.50	2.77 ± .87 p <.001	99 ± 13.71 p <.01
	Ox bile salts, 30 mg/200 g intraper. inj.	19	175 ± 21.70	1.10 ± .59 p <.001	59 ± 26.55 p <.001

* Mean ± stand. dev. † 6 hr collection of gastric juice in Group 3. p = Probability of significance of t value.

ene tube and leading the tube to the outside *via* a stab wound. The duct was ligated below point of insertion of the tube. In controls the tube was implanted into the duodenum. In rats with external biliary drainage, the 4 hour gastric output was a little higher than in controls (Table I).

Group 3. Because of longer duration of anesthesia in Group 2, removal of bile from the intestine was achieved in another manner. Twenty-four hours prior to pyloric ligation, the bile duct was shunted to the rectum (Fig. 1). A sham operation was done in controls. There was no difference in gastric secretion between bile shunted rats and controls (Table I).

Group 4. Simultaneously with pyloric ligation, a fine polyethylene tube was placed in the tail vein and the latter infused continuously during 4 hour post operative period with 20% solution of sodium dehydrocholate. The infusion speed was adjusted so that a total dose of 150 mg/200 g of sodium dehydrocholate was given. Controls received an equal volume of normal saline. There was inhibition of volume and free HCl concentration of gastric content in the treated rats (Table I). Lower doses of sodium dehydrocholate or of ox bile salts administered by this route produced only inconsistent inhibition.

Group 5. Treated rats received 20 or 30 mg of ox bile salts in 2 ml of normal saline as single intraperitoneal dose at time of pyloric ligation. Controls received the same volume of normal saline. Consistent inhibition of same parameters occurred at these dosage levels (Table I). Doses higher than 30 mg/200 g resulted in total inhibition whereas doses lower than 20 mg/200 g resulted in inconsistent inhibition and only of volume of gastric content.

Discussion. These data suggest that gastric inhibition produced by bile duct ligation in the rat can be attributed to increase in level of circulating bile salts. This is known to be one of the effects of bile duct obstruction. The lesser inhibitory activity of intravenously administered bile salts may be explained by the fact that it is difficult to increase the level of circulating bile salts by intravenous injection unless huge doses are given. This appears to be due to rapid uptake by liver and adsorption by tissues and blood vessel walls(6). Presumably, this inhibitory effect of bile salts on

FIG. 1. Proximal portion of bile duct catheterized with fine polyethylene tube. Duct is ligated below point of insertion of tube. Free end of cannula is lead through mesentery of duodenum and implanted into rectum. With this technic entire bile output is diverted from small intestine and coecum. Pancreatic secretions reaching intestine via distal segment of bile duct are left undisturbed.

gastric secretion is related to increase in level of bile salts circulating in the pool intestine-portal blood-liver, and intraperitoneal administration should therefore be more effective. Amounts administered (23-30 mg/200 g) do not appear excessive in view of the fact that the rat excretes approximately 10-17 mg of cholic acid/hour(7) in the bile. At present, the mechanism by which parenterally administered bile salts inhibit gastric secretion in the rat cannot be explained. That this does occur is interesting since bile salts are naturally occurring compounds. Increased resorption of bile salts during digestion and absorption of food in the intestine may play a part in the physiological mechanisms of gastric inhibition following gastric emptying.

Summary. Gastric secretory activity (volume and free HCl concentration) in the pylorus ligated rat is inhibited by bile duct ligation. This is not due to removal of bile from the intestine but appears to be due to increase in level of circulating bile constituents, particularly bile salts, resulting from obstruction to the outflow of bile from the liver.

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Urinary Metabolites of Chlorpropamide in Dogs, Rabbits, and Man.* (25055)

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During pharmacological investigation of chlorpropamide [1-(*p*-chlorobenzenesulfonyl)-3-n-propylurea](1), a hypoglycemic agent, urinary excretion of the drug was measured by the method of Spingler(2). From 30 to 40% of orally administered chlorpropamide was detected in dog urine. This suggested that more than 50% of the ingested dose was metabolized. This report is concerned with identification of chlorpropamide metabolites in dog, rabbit, and human urine.

* The authors are deeply indebted to D. O. Woolf for infrared absorption curves, to Ann VanCamp for x-ray diffraction patterns, and to Dr. R. E. McMahon for sublimations.

Methods. Twenty-four hour urine speci-

mens taken after oral chlorpropamide administration were acidified to pH 2 and extracted 16 hours with methylene chloride in a continuous extractor. The methylene chloride solutions were evaporated and the residue dissolved in methanol in preparation for paper chromatography. Aliquots of the methanol solution were spotted on Whatman No. 1 filter paper and developed 16 hours at room temperature in an ascending manner with butanol saturated with 5 N ammonium hydroxide. The sheets were dried at room temperature, then immersed in freshly prepared saturated aqueous solution of mercurous nitrate according to the method of Deininger (3). After one minute of rinsing in distilled water the sheet was examined by placing it on glass plate. This permitted location of major spots by observation of the reduced translucence of the wet paper. The appearance of a gray spot as the result of reaction of sulfonamides with mercurous nitrate could also be observed. To make the spots more visible the wet sheet was slowly moved through 1 : 3 acetone dilution of saturated acetone solution of *p*-dimethylamino-benzalrhodanine. The spots then appeared reddish brown against a yellow-brown background. The sheet was then dried at room temperature. The quantity of compounds was estimated by spectrophotometry. After detection of the spots, the corresponding areas of an adjacent lane were cut out for elution. Elution was accomplished by immersing the section under investigation in .01 N sodium hydroxide solution and slowly agitating it for one hour. Ultraviolet absorption curve was checked against that of model compound of like mobility, chromatographed, and eluted in the same way. Only those runs in which the unknown absorption curve was identical with that of the known were considered in the quantitative determination. For isolation of metabolites, urine extracts were spotted on Whatman No. 1 or 3 MM paper and developed as before. Horizontal strips were cut out corresponding to the R_f of various spots as determined by mercurous nitrate-*p*-dimethylaminobenzalrhodanine treatment. These horizontal strips were eluted with methanol, the eluates were evaporated, and infrared absorption curves of the residue determined. Crystalline products were identified by x-ray powder diffraction methods.

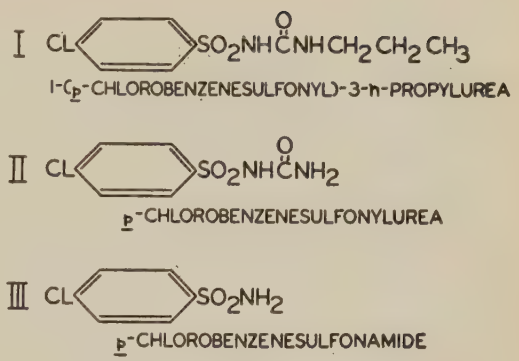

FIG. 1. Urinary excretion products of chlorpropamide.

Results. 1. *Dog.* Paper chromatograms of chloroform or methylene chloride extracts of urine from normal dogs receiving daily oral doses of chlorpropamide were compared with extracts from normal untreated dogs. Three additional spots (R_f 0.82, 0.70, and 0.42) were produced by extracts from treated dogs. The most mobile of these spots appeared gray after the mercurous nitrate reaction while the other 2 appeared as white precipitates. All 3 were clearly visible after reaction with *p*-dimethylaminobenzalrhodanine.

Chlorpropamide had an R_f of 0.71 in this system, therefore the middle spot was attributed to unchanged drug.

Two possible metabolites, (Fig. 1) *p*-chlorobenzenesulfonylurea (II) and *p*-chlorobenzenesulfonamide (III), were made available by Dr. M. V. Sigal, Jr. of these laboratories. When these compounds were chromatographed, the sulfonamide (III) gave a gray spot (R_f 0.82) with mercurous nitrate and sulfonylurea (II) had a mobility corresponding to slowest moving of the 3 spots in question (R_f 0.42).

A quantitative determination of the 3 concentrations as they occurred after chromatographic development was made by determination of ultraviolet absorption of the eluant of each area at the absorption maxima observed for each of corresponding model compounds. Twenty-four hour urine samples from a dog maintained on daily oral dose of 0.5 g of chlorpropamide contained from 27 to 33%

of administered dose as unchanged drug. The sulfonylurea (II) represented 35 to 40% of the dose, and the sulfonamide (III) an additional 16 to 24%, giving a total recovery from 78 to 97% of administered drug.

For additional identification of the 3 urinary excretion products, the infrared absorption curves of eluants from appropriate areas of paper chromatograms of urine extracts were compared with curves of model compounds and were in agreement in each case. Crystals from a benzene solution of the most mobile spot (R_f 0.82), from sublimation under vacuum at 110°C for the next most mobile spot (R_f 0.70), and from an alcohol: water solution of the least mobile spot (R_f 0.42) had x-ray powder diffraction patterns identical with the model compounds crystallized under same conditions.

2. *Rabbit.* A 24-hour urine sample from normal rabbit that had received 100 mg of chlorpropamide orally was extracted and chromatographed. One large spot appeared in addition to those attributable to normal rabbit urine constituents. This spot had the same mobility as chlorpropamide. The ultraviolet absorption spectrum of the eluant was identical with that of chlorpropamide. The 24-hour excretion was 86 mg or 86% of the dose as unchanged drug. The fate of remaining 14% has not been determined.

3. *Man.* Twenty-four hour urine samples from 3 diabetic patients who had received 0.3 to 1 g of chlorpropamide orally were extracted and chromatographed. Spots corresponding to sulfonamide (III) and the unchanged drug (I) were seen. The sulfonylurea spot, if present, was difficult to distinguish because of interfering substances present in human urine. One of the subjects received 1 g of chlorpropamide on the first, second and fifth day of treatment. Only urine specimens 24 hours subsequent to each dose were available for investigation. The extract from final of these specimens produced a spot (R_f 0.55) that has yet not been identified. The *p*-chlorobenzenesulfonamide was identified by x-ray powder diffraction pattern as in the dog. The infrared absorption curve from the supposed chlorpropamide was identical with chlorpropamide except for some additional absorption that was probably due to carbonyl contamination. A sublimation attempt yielded sulfonamide (III) instead of the expected unchanged drug. No sulfonylurea has been identified.

Discussion. Excretion of chlorpropamide and its metabolites in dog urine accounted for 78 to 97% of amount of drug administered daily. The 2 degradation products of chlorpropamide, *p*-chlorobenzenesulfonylurea (II) and *p*-chlorobenzenesulfonamide (III), correspond to *p*-tolylsulfonylurea and *p*-tolylsulfonamide isolated by Mohnike *et al.*(4,5) from tolbutamide-treated dogs.

The recovery of 86% of the unchanged drug in the rabbit 24 hours after a single dose is evidence that chlorpropamide is resistant to metabolism in this species.

Johnson *et al.*(6), using S^{35} labeled chlorpropamide, reported no apparent metabolic alteration of the drug in humans. In this investigation of urinary excretion products following chlorpropamide therapy in man, it has been shown that metabolic degradation does occur. One metabolite has been isolated and identified as *p*-chlorobenzensulfonamide. It is possible that *p*-chlorobenzenesulfonylurea is also present but sufficient confirmatory data are not available. The paper chromatograms showed an additional metabolite that has not been identified. Excretion of chlorpropamide *per se* was proven by paper chromatography and infrared absorption spectra. Failure to get crystalline material was probably due to small amount of water in the eluate which caused degradation of chlorpropamide to sulfonamide during sublimation.

Summary. Metabolism of 1-(*p*-chlorobenzenesulfonyl)-3-n-propylurea was investigated in dog, rabbit and man. The rabbit excreted more than three-fourths of the drug without chemical alteration. Less than half of orally ingested drug was excreted unchanged by the dog, most of the remainder appeared as *p*-chlorobenzenesulfonylurea and *p*-chlorobenzenesulfonamide. As the result of a limited investigation in man, unchanged chlorpropamide and *p*-chlorobenzenesulfonamide were found in the urine. One other possible metabolite was indicated by paper chromatograms.

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Effect of Triiodothyronine and Cortisol on Intracellular Penetration of D-xylose. (25056)

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Studies with uncut rat diaphragm preparation demonstrated the *in vitro* effectiveness of insulin in permitting entry of D-xylose into a muscle fiber water space otherwise unavailable to sugar for distribution(1,2,3). To determine whether other hormonal treatments influence sugar penetration in this preparation, the effect of epinephrine, cortisol, and triiodothyronine (TIT) administered *in vitro* and *in vivo* have been examined. Since it was reported that metabolic poisons increase sugar penetration(2), it was of interest to learn if depletion of energy reserves *via* starvation would influence volume of distribution available to sugar.

Methods. The uncut rat diaphragm preparation and technics employed for pentose and sucrose determination were previously described(3). Incubation medium contained 128 meq/l-NaCl, 5.1 meq/l-KCl, 2.72 meq/l-$CaCl_2$, 1.02 meq/l-$MgSO_4$, 20 meq/l-$Na_2HPO_4/NaHPO_4$ buffer system (pH 7.2) and D-xylose at 4 mg/ml ± glucose at 1 mg/ml, or sucrose at 8 mg/ml. For *in vitro* experiments, TIT* was used as sodium salt (dissolved in 0.13 N-NaOH) at final concentration of 6 μg/ml medium. For *in vivo* administration, 300 μg TIT/day/rat were given ip 4 days. Epinephrine HCl was used *in vitro* at concentration of 2 μg/ml. Cortisol† was used *in vitro* at 40 μg/ml in 1% Tween 80 or *in vivo* at dosage regimen of 2 mg/day/rat ip. Insulin‡ was used at 0.4 units/ml (16 μg/ml) *in vitro.* Incubations were carried out in 50 ml medium volume at 38°C with shaking (90 cycles/min. amplitude 8 cm). Following incubation, diaphragm muscle was removed from rib cage, wiped across clean glass plate, weighed, and placed into a tube containing 2 ml distilled H_2O from which xylose or sucrose was extracted and determined. Sugar distribution in tissue was expressed relative to concentration in the medium. All animals were fed *ad lib.* except in experiments where 24 hr or 56 hr starvation period is indicated. Incubations were carried out for 60 and 90 minute periods since previous kinetic studies have shown that equilibration is attained in available water space within 60 minutes.

Results. Table I shows results obtained following *in vitro* or *in vivo* administrations of epinephrine, triiodothyronine, cortisol and/or insulin or starvation. These findings may be summarized as follows:

Triiodothyronine. Addition of TIT to incubation medium had no demonstrable effect upon distribution of D-xylose during 60 or 90 minute incubation. However, following parenteral administration of TIT, distribution of D-xylose is increased over controls. Treatment with TIT produced no change in total tissue water (79.1 ml/100 g ± 0.6) or in the sucrose space which may be assumed a measure of extracellular space. Thus increase in tissue xylose would seem to result from increased intracellular penetration. Furthermore, when diaphragms of TIT treated animals are incubated with insulin *in vitro,* the

* L-Triiodothyronine generously supplied by Dr. A. E. Heming, Smith, Kline & French Laboratory.

† Cortisol generously supplied by Dr. R. H. Silber, Merck Inst. for Ther. Res.

‡ Insulin was generously supplied by Dr. E. Rohrman, Eli Lilly & Co.

TABLE I. Sugar Distribution in Uncut Rat Diaphragm.

Treatment	Xylose, ml/100 g (wet wt)	Sucrose, ml/100 g (wet wt)	Xylose, ml/100 ml intracellular H_2O
Control	37.1 ± .45*(22)†	22.3 ± 1.0*(4)†	26.1
Insulin *(in vitro)*	59.3 ± .78 (29)	22.5 ± 1.1 (4)	64.8
Triiodothyronine *(in vitro)*	37.5 ± 1.6 (6)	22.7 ± .9 (3)	26.1
Triiodothyronine *(in vivo)*	48.1 ± 1.53 (12)	21.5 ± 1.2 (4)	47.0
Idem + insulin *(in vitro)*	64.4 ± .73 (12)	21.9 ± 1.0 (4)	75.0
Epinephrine *(in vitro)*	36.2 ± 1.8 (6)		
Idem + insulin *(in vitro)*	60.1 ± 2.3 (4)		
Cortisol *(in vitro)*	37.4 ± 1.2 (6)		
Cortisol *(in vivo)*	44.4 ± 1.62 (11)	24.4 ± 1.1 (4)	35.3
Idem + insulin *(in vitro)*	63.9 ± 1.33 (12)	24.2 ± 1.0 (4)	70.0
56 hr starvation	48.6 ± 1.6 (10)	23.4 ± 1.2 (4)	44.4
Idem + insulin *(in vitro)*	70.0 ± 1.7 (6)	23.2 ± 1.4 (3)	82.3
24 hr starvation	37.7 ± 1.4 (8)	22.3 ± 1.0 (4)	27.1
Idem + insulin *(in vitro)*	60.6 ± 1.6 (4)	22.6 ± 1.1 (4)	67.0

* Mean, stand. error of mean. † No. of determinations.

intracellular xylose space is greater than observed with insulin in diaphragms from non-treated rats. Thus xylose occupies 65% of total intracellular water in normal diaphragms whereas following TIT treatment, xylose distributes in 75% of cell water. The difference is statistically significant ($p<0.01$).

Cortisol. Cortisol, like TIT, had no influence on distribution of D-xylose when added *in vitro*. Following parenteral injection, an creased distribution of D-xylose was found both in absence and presence of exogenous insulin. Unlike triiodothyronine however, cortisol appears to increase the sucrose space from 22% to 24% of tissue weight. When this larger figure is used to calculate intracellular penetration, an increase from 26% to 35% is found in absence of insulin and 65% to 70% in its presence.

Epinephrine. Epinephrine, *in vitro,* was completely without effect on pentration of D-xylose in presence or absence of added insulin. In view of rapid onset of action which this endocrine factor exhibits, our study does not include parenteral administration of epinephrine since a latent period of action exceeding 90 minutes was not considered a likely possibility.

Starvation. From these results it becomes apparent that either interference with endogenous metabolic pathways by use of metabolic poisons(2), or previous depletion of endogenous "energy-substrate" decreases capacity of intracellular water to exclude sugar and in this way an "insulin-like" effect is obtained. Starvation for 56 hr raised the xylose space from 37.1 ml/100 g in non-fasted controls to 48.6 ml/100 g. In presence of insulin, a distribution of 70.3 ml/100 g was observed which is in excess of the usual 59 ml/100 g found with non-fasted animals. Thus, in effect, starvation treatment produces an almost quantitatively additive penetration of sugar in presence and absence of insulin. Addition of glucose at 1 mg/ml to the incubation medium was without effect on xylose space measured under all conditions employed in these studies.

Discussion. It has been shown that the properties of exclusion which cell water of diaphragm muscle fiber exhibits, can be appreciably altered by a number of conditions apart from and concomitant with insulin treatment. That prolonged starvation readily breaks down such penetration barriers strongly indicates that the system for maintenance of these barriers is energy dependent. This is in good agreement with the work of Randle and Smith using metabolic poisons (2) and with previous findings of intracellular sodium distribution(4). Since both triiodothyronine and cortisol do allow entrance of sugar into an intracellular water space, normally unavailable, the question arises as to whether such action may be involved in the physiological role played by these 2 sub-

stances. If one makes the assumption that sucrose space is a measure of extracellular space (3) then these results show that in the absence of exogenous insulin, triiodothyronine treatment gives sugar access to intracellular water space of some 20 ml/100 ml greater than control values and in the presence of exogenous insulin, an access to water space 10 ml/100 ml greater than that afforded by insulin.

These results with xylose are representative of those with other monosaccharides such as glucose, for the flux of D-xylose which is equivalent to that of D-galactose, closely approximates values obtained for glucose entry as measured by glucose disappearance at physiological 1 mg/ml glucose concentration level(3). However, if a water space which previously excluded glucose or xylose now is penetrable by these sugars, it may now well be penetrable by other suitable substrates which previously were not readily able to gain entrance. If this effect of triiodothyronine and cortisol upon sugar penetration is a physiologically qualitative action, and exogenous treatment merely exaggerates the quantitative values much as exogenous insulin has been shown to do, then one would have an explanation in general terms on cellular level of how a hormone, *e.g.*, insulin, thyroid, or cortisol, may act. This would be to regulate availability of the substrate, whether it be sugar or other, for the various enzymic systems within the intracellular water. However, since other factors which impair cellular energetics also increase available cell water, there is the possibility that the effects of TIT and/or cortisol on sugar penetration merely reflect the variable degree of protein catabolism which they invoke. Furthermore, the specificity of this response on sugar penetration remains to be determined. There is the possibility that the effects observed are merely nonspecific pharmacologic actions and that substances of similar chemical structure might yield similar results. Work is currently under way using chemical analogs of those employed, other steroid hormones and some trophic factors of the anterior hypophyseal lobe.

There remains the question of whether this effect of triiodothyronine or cortisol on sugar penetration is exerted directly upon muscle, or if it is the result of one or more intermediary events which in turn produces the substance capable of acting upon the muscle. Lack of an *in vitro* response with either of these endocrine factors is not too informative since a period of much greater than one hour may be required for the direct effect to be manifested. With epinephrine, however, *in vitro* effects are rapid, and absence of a response here is taken to mean that its anti-insulin action *in vivo* does not in any way involve sugar transfer. Preliminary work of a somewhat different kind with triiodothyronine has demonstrated the effect herein reported to be a direct one in seemingly unequivocal terms. Details of this latter work will be published.

Summary. Distribution of D-xylose in the uncut rat diaphragm was studied *in vitro* under a variety of conditions. It was found that epinephrine, triiodothyronine or cortisol had no effect on distribution of this sugar when administered *in vitro*. Triiodothyronine or cortisol, administered parenterally, increased the space occupied by D-xylose in absence of insulin, and in presence of insulin it resulted in comparatively small but consistent addition to the space occupied when insulin alone was used. A 24-hour starvation period was without effect on sugar distribution whereas a 56-hour period increased the xylose space in absence of insulin and was quantitatively additive in its presence.

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Distribution of Carbonic Anhydrase in Alligator. Effect of Acetazolamide on Blood and Aqueous Humor CO_2. (25057)

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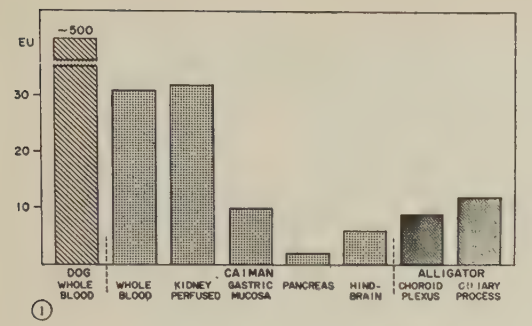
In the alligator the carbonic anhydrase inhibitor acetazolamide causes a more acid urine and a tendency toward alkalemia(1,2), an effect opposite to that seen after inhibition of carbonic anhydrase in mammalian kidney (3,4). It has been suggested that "renal carbonic anhydrase is necessary for conservation of chloride by the alligator"(2). As there seems to be no information on distribution of carbonic anhydrase in the reptile, it seemed interesting to see whether the enzyme is present in the reptile kidney and, whether distribution of carbonic anhydrase in tissues of these animals differs from that in the mammal. Qualitative differences in enzyme distribution among different species have been reported: *i.e.* in dogfish, *Squalus acanthias,* lens and kidney contain no carbonic anhydrase(5,6) in contrast to the mammal. It was also of interest to see whether CO_2 content of another carbonic anhydrase dependent secretion, *i.e.* aqueous humor, is changed in the same way as in the mammal following carbonic anhydrase inhibition.

Materials and methods. Ten caimans, (*C. laitrostris,* a South American alligator) averaging 90 g weight and 35 cm length, were fasted 3-4 days before experiments, but had access to water, at room temperature, 25°C ± 1. Experiments were done in April and May. A female alligator (*A. mississipiensis*) weighing 21 kg and measuring 160 cm was maintained in similar fashion to the caimans. Blood was obtained by heart puncture and aqueous humor, 0.7 ml from one eye at a time, was taken from anterior chamber. Acetazolamide, N.N.D., a carbonic anhydrase inhibitor (10% solution, Diamox®) was given intracardially, 50 mg/kg. A micromethod adaptation(7), scaled down 10-fold, of the method of *Philpot* and *Philpot*(8) was utilized in analysis of aliquots for presence of carbonic anhydrase. Tissues were weighed, diluted with double distilled water, and ground in glass homogenizer. The entire kidney, pancreas, and lens were used; gastric mucosa was dissected from the muscularis; the brain was stripped of vessels and cut just anterior to cerebellum; cortical tissue was removed from anterior brain before analysis; the choroid plexus was removed from the anterior brain; the ciliary processes were cut from the underlying connective tissues. After administration of acetazolamide, blood and aqueous humor samples were drawn anaerobically; pH determined (Cambridge pH meter, model R) at cloacal temperature of the reptile and CO_2 determined by micro Van Slyke (Microgasometer, model 600; Kopp-Natelson) method. The content of acetazolamide in plasma, saline washed red cells, and aqueous humor was determined by enzymatic method of *Maren et al.*(7).

Results. The enzyme results are tabulated in Fig. 1 and the inhibitor experiment is shown in Fig. 2. Distribution of carbonic anhydrase was qualitatively similar but quantitatively less than in the mammal(9) with the exception of the lens, where no activity was found. Such activity has, however, been reported in the chicken lens(10). The role of the enzyme in the lens is not understood(11).

The finding of carbonic anhydrase activity in the kidney explains the effects on composition of urine, observed following administration of carbonic anhydrase inhibitor(2). The comparatively long lasting effect (4 days) on alligator kidney, following acetazolamide, is explained by the very slow disappearance of the drug from plasma; the plasma half life was about 40 hours compared to 1½ hours in man. Sequestration of the drug into red cells is similar to that seen in the mammal (12). All of these pharmacological characteristics of acetazolamide are similar to those seen in *Sq. acanthias*(6). The steady state ratio of the acetazolamide concentration in the aqueous over that in plasma was about 0.8 compared to 0.05 in the rabbit(13).

The CO_2 content of reptile blood varies con-



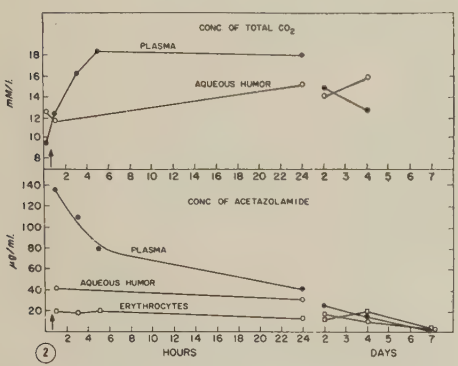


FIG. 1. Distribution of carbonic anhydrase in the caiman and alligator. Abscissa: different tissues. Each column represents avg value of tissues from 4 animals, except for the alligator where the column represents one animal. Ordinate: enzyme units (EU) as defined by Maren *et al.*(7).

FIG. 2. Distribution of acetazolamide in plasma, erythrocytes and aqueous humor following 50 mg/kg intracardially, to the alligator, and effect of this inj. on CO_2 content of plasma and aqueous humor.

siderably with season(14) and body temperature(15). It is also changed by food intake and excitement(16,17). Our values for plasma CO_2 (Fig. 2) are comparatively low. The sharp rise in plasma total CO_2 (Fig. 2) following acetazolamide has been seen in another species, the dogfish(5,6). This rise of plasma CO_2 is probably due to inhibition of both blood and kidney carbonic anhydrase in the alligator, but in the dogfish only to inhibition of the blood-gill system.

Total CO_2 of aqueous humor was higher than that of the plasma (Fig. 2), also seen in some mammals(18). Following acetazolamide, not much of a change was seen in the aqueous total CO_2, in spite of the rise in plasma, causing ratio of total CO_2 in the aqueous humor to that in plasma to fall considerably. The drop of this ratio is also seen in some mammals(19) but in those cases (*cf.* rabbit) the change is caused by more rapid drop of aqueous humor CO_2 as compared to that of plasma.

It is not possible from these experiments to tell whether acetazolamide changes the local production of CO_2 into the aqueous humor of the alligator, since such an effect could be masked by the simultaneous change of plasma CO_2.

Summary. Carbonic anhydrase was found in the caiman (*C. laitrostris*) erythrocytes, kidney, gastric mucosa, pancreas and hindbrain. No activity, however, was found in the lens. The enzyme was also found in the alligator (*A. mississipiensis*) choroid plexus and ciliary processes. Carbonic anhydrase inhibition in the alligator caused a long-lasting rise of plasma total CO_2 but no certain change of anterior aqueous total CO_2 was observed.

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Device to Control Constriction of Main Renal Artery for Production of Hypertension in Small Animals.* (25058)

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Various methods have been employed for production of experimental renal hypertension in the rat, but most of them have drawbacks. For small animals, the production and application of the original silver clamp devised for constriction of the main renal artery in the dog and other large animals(1) are impractical. The simplest method is the constriction of the main renal artery by a silver clip, or band(2-4). This method lacks adequate means of controlling degree of constriction of the artery. To this end, an instrument has been constructed with which this constriction can be made uniform and which facilitates production of acute and chronic hypertension in a high percentage of rats of equal weight. The instrument was fashioned from old type surgical needle holder. Through one jaw of instrument (Fig. 1, A) a screw (B) is inserted, the end of which comes in contact with inner surface of opposite jaw (C). Adjustment of this screw keeps the jaws apart to any desired extent, and turning of screw in one or the other direction controls the space left between the jaws, the width of which can be definitely determined. This is done by closing the jaws of forceps on blades of a mechanic's feeler gauge, graduated in fractions of an inch, in such a way that, when the screw touches the opposite jaw, the desired gauge blade can be slipped snugly into the space. By testing the effect of the forceps on a gauge blade of next size, larger and smaller, a check on correct adjustment can be

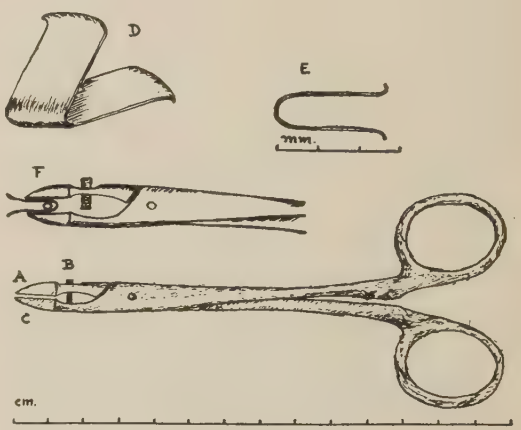

FIG. 1.

made. The jaws of the instrument must be rigid toward the tip, and the surfaces which oppose one another must be machined perfectly flat and smooth. The silver clip (Fig. 1, D & E) for constriction of main renal artery is made from a strip of annealed, rolled, silver ribbon 2 mm wide and 0.127 mm thick. This strip is divided into sections measuring 6 mm in length, and the edges are filed round and smooth. The clip is then bent into a U-shape, over an ordinary pin, and the 2 ends are bent slightly outward (Fig. 1, E), to facilitate application.

Application of clip. Through a costo-lumbar incision, with kidney retracted toward the abdomen, the renal pedicle is exposed, the artery is dissected clean, and the clip is slipped around it, near the aorta. The free edges of the clip are then approximated by means of a hemostat, to enclose the artery. With the jaws of instrument previously adjusted to desired gauge, about half of the "U" of the clip, including the rounded end, in which the ar-

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† I wish to thank Dr. Erwin Haas for advice and Louise Fingers for technical assistance.

tery rests (Fig. 1, F), is grasped in the jaws of the forceps and compressed firmly so as to constrict the vessel to the desired degree.

It has been found by experience that for female rats, weighing about 150 g, constriction of only one main renal artery, with the forceps set to leave a space of 0.014″ (or 0.355 mm) leads to development of hypertension in the majority of animals. The wall of the renal artery is actually compressed by this procedure to outside thickness of about 0.1 mm. Greater constriction, which leads to complete necrosis of the kidney, or inadequate constriction, is ineffective in raising the blood pressure. For female rats weighing around 200 g, a free space of 0.015″ is necessary, and for those weighing around 250 g, a free space of 0.016″ is most satisfactory. In the case of rapidly growing young male rats, a degree of constriction, adequate for a young female rat of equal weight may prove to be too tight and eventually lead to necrosis of the kidney, as the animal grows. This must be taken into consideration.

Summary. An instrument has been described which permits accurate control of degree of constriction of the main renal artery in small animals and facilitates production of hypertension in a large percentage of such animals.

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Metrotropic Activity of Some 21-Haloprogesterone Derivatives.* (25059)

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Recent interest in obtaining orally active progestational agents resulted in synthesis of many structural modifications of progesterone and 19-nortestosterone. Certain of these materials have been shown to be of value clinically. In the progesterone series, metrotropic activity has been reported for a series of 9-α and 12-α-halogenated derivatives of progesterone(1). 9α-Bromo-11-oxoprogesterone was effective clinically upon oral administration; its potency was rated as equal to that of 17α-hydroxyprogesterone acetate and about 2 to 3 times more potent than ethisterone(2,3). 9α-Fluoro-11β-hydroxyprogesterone and its 17α-hydroxy analogue were weak metrotropic agents in rabbits(4). These same agents were reported to be more active than progesterone as antagonists of estrone-induced uterine growth in rats(5). In a similar test 9α-fluoro-11β-hydroxy and 9α-fluoro-11-oxoprogesterone were about equal to progesterone(1). The present communication deals with progesterone-like activities and structure-function relationships of several 21-halogenated steroids.

Materials and methods. The following steroids were used: Progesterone, 21-fluoroprogesterone, 21-chloroprogesterone, 17α-hydroxyprogesterone, 17α-hydroxy-21-fluoroprogesterone, 17α-hydroxy-21-chloroprogesterone, 17α-acetoxyprogesterone, 17α-acetoxy-21-fluoroprogesterone, 17α-acetoxy-21-chloroprogesterone, 17α-acetoxy-21-bromoprogesterone, 17α-acetoxy-21-iodoprogesterone, 6α-methyl-17-acetoxy-progesterone, 6α-methyl-17-acetoxy-21-fluoroprogesterone and 6α-methyl-17-acetoxy-21-chloroprogesterone. Estimates of activity were obtained using the Clauberg assay, uterine carbonic anhydrase assay and the intra-uterine (McGinty) assay. *Clauberg Assay*(6). The test compound was administered subcutaneously or orally over 5 days to immature female rabbits previously primed for 6 days with 5 μg estradiol-17β. On day following last treatment, animals were sacrificed and

* The new steroids described in this study were prepared by Drs. C. G. Bergstrom, P. B. Sollman and R. M. Dodson, Division of Chemical Research, G. D. Searle & Co.

segments of uteri taken for histological inspection. On microscopic examination arborization of glandular epithelium was determined and graded from +1 to +4; +1 represented that type of uterus with no proliferation and +4 represented maximally stimulated epithelial glands. *Uterine carbonic anhydrase assay.* Uterine tissues weighing approximately 100 mg were removed from animals used in Clauberg assay. Tissues, which included both endometrium and myometrium, were homogenized using cold distilled water and centrifuged; the supernatant was analyzed for carbonic anhydrase using our modification of methods of Philpot and Philpot(7) and Keilen and Lutwak-Mann(8). Briefly, our method was as follows: 5 ml aliquots of supernatants were placed in reaction tubes along with 5 ml of a 0.0026 M $NaHCO_3$ solution, 10 drops bromothymol blue and 1 drop octyl alcohol; all solutions and subsequent reactions were carried out at 0-2°C. Blanks consisted of 10 ml 0.0026 M $NaHCO_3$ in addition to bromothymol blue and octyl alcohol. CO_2 was bubbled through solutions for exactly 2 minutes, the bubble rate regulated by pressure gauge and capillary tubing so that a color change in blank tubes occurred in approximately 75-80 seconds. At the end of 2 minute bubbling period 1 ml of a $NaCO_3/NaHCO_3$† solution was added and the reaction required to change the color of bromothymol blue from blue to blue-green was carefully timed. The end-point color was prepared using 10 ml buffer (Beckman #3501, pH 7) solution with 10 drops of the indicator. Units of activity were estimated with the following calculations, based on standard curves obtained with purified enzyme preparation (National Biochemical Corp.):

$$x = \frac{P}{100} - P \cdot X_{50} \cdot \frac{\text{Vol extract}}{\text{Vol ext. used}} \cdot \frac{100}{\text{Tissue wt}},$$

where x = weight of crystalline enzyme (or its equivalent activity in 100 mg tissue); X_{50} = amount of crystalline enzyme producing 50% reduction of reaction time; P = Per cent reduction in reaction time (RT)

$$= \frac{\text{RT blank} - \text{RT unknown}}{\text{RT blank}} \cdot 100.$$

McGinty assay(9). Ovariectomized estrone-primed rabbits were used. The compound, dissolved in corn oil, was administered locally in a one-inch segment of one horn of uterus; a similar segment of the contralateral horn of the uterus was treated with oil only. Three days later, animals were sacrificed and uterine segments removed and prepared for histological inspection as in the Clauberg technic.

Results. Agreement between Clauberg and uterine carbonic anhydrase assays is in keeping with earlier reports by others(10,11,12). Sensitivity and reproducibility of the enzyme test are about the same as with the Clauberg test even though no attempt was made to dissect the endometrial portion of the uterus away from the myometrium, as was done in the methods previously cited.

17α-Hydroxyprogesterone, inactive as a progestin in rabbits(13,14,15,16), guinea pigs (17) and humans(16) is highly active in mice (Hooker-Forbes Assay, 18). The marked activity found following 17α-acetylation of this compound (Table I) confirms that reported by others(3,12,13,14,15,19). Presence of 6α-methyl group in the progesterone molecule increased its potency 5-fold on subcutaneous administration (Table I). Orally, 6α-methylprogesterone was as effective as 17α-acetoxyprogesterone (Table II). The most potent metrotropic agent was 6α-methyl-17α-acetoxy-21-fluoroprogesterone, which was 50 times more potent than progesterone on subcutaneous administration. Orally, 6α-methyl-17α-acetoxy-fluoroprogesterone was 2 times more potent than its non-halogenated analogue and 10 times more potent than the standard, subcutaneously administered progesterone (Table II). This agent was also the most potent progestin of the present series in the McGinty assay, being about 50 times more potent than progesterone (Table III).

Systemically, 21-chloroprogestins were less potent than their C-21 fluoro-derivatives (Table I). The C-21 bromo- and iodo-substituted forms tested had only minimal activity, or were inactive, suggesting an inverse relationship between progestational activity and

† The reaction solution is prepared for each determination by adding 5 ml conc. $NaHCO_3$ to 25 ml conc. Na_2CO_3 and diluting to 100 ml with double distilled water.

TABLE I. Metrotropic Activity of C-21 Haloprogestins in Clauberg and Uterine Carbonic Anhydrase Assays Following Subcutaneous Administration.

Progesterone substitution			Clauberg assay			Carbonic anhydrase assay		
C-6	C-17	C-21	Daily dose (mg)	No. of animals	Relative activity*	Daily dose (mg)	No. of animals	Relative activity*
			.1	32	1	.1	32	1
		F	.05	4	2			
		Cl	1.	4	Inactive			
	OH		5.	4	"	5.	4	Inactive
	"	F	1.	4	"	1.	4	"
	"	Cl	1.	4	"	1.	4	"
	OAc		.01	8	10	.01	8	10
	"	F	.01	4	10			
	"	Cl	.05	4	2	.05	4	2
	"	Br	.1	4	1	.1	4	1
	"	I	1.	3	Inactive			
CH_3			.02	4	5	.02	4	5
"	OAc		.002	8	50	.003	8	30
"	"	F	.002	15	50	.002	14	50
"	"	Cl	.04	8	2	.04	8	2

* The lowest daily dose that produced a +3 endometrial response was arbitrarily chosen as the level at which potency comparisons were made in the Clauberg Assay. In the carbonic anhydrase assay comparisons were made at level that corresponded to 50 μg equivalents/100 mg wet wt of tissue.

atomic weight of the substituted halogen. In absence of adequate knowledge concerning metabolism of these substances, any explanation of structure-function relationships must be speculative. The increased oral effect of the 21-fluoro-progestins apparently results from decreased degradation in the gut or increased intestinal absorption since their parenteral activities are not different from those of their non-halogenated forms (Tables I and II). Conversely, the decrease in potency of the 21-chloro-, bromo-, and iodo-progestins when compared to the 21-fluoro-derivative may result from increased degradation of the molecule in the gastro-intestinal tract. Increased systemic inactivation can not be ruled out.

With the exception of 17α-acetoxy-21-fluoroprogesterone, similar structure-function relationships obtain following local instillation of these 21-haloprogestins (Table III). Based upon the relationship between metrotropic activity and atomic weight of the substituted halogen, the relatively low potency of 21-

TABLE II. Metrotropic Activity of C-21 Haloprogestins in Clauberg and Uterine Carbonic Anhydrase Assays Following Buccal Administration.

Progesterone substitution			Clauberg assay			Carbonic anhydrase assay		
C-6	C-17	C-21	Daily dose (mg)	No. of animals	Relative activity*	Daily dose (mg)	No. of animals	Relative activity*
			.1	32	1	.1	32	1
		F	1.	4	Inactive†			
		Cl						
	OH		5.	4	"	5.	4	Inactive
	"	F	1.	4	"	1.	4	"
	OAc		.5	3	.2	.5	3	.2
	"	F	.1	7	1.			
	"	Cl	.5	6	<.2	.5	6	<.2
	"	Br	1.	4	Inactive†	1.	4	Inactive
CH_3			.5	4	.2	.5	4	.2
"	OAc		.02	5	5.	.02	5	5.
"	"	F	.01	8	10.	.01	8	10.

* Subcut. administered progesterone was used as standard for comparison, since its oral activity is so low.

† Active, but not by standards of this study, since the compound did not produce a +3 response.

TABLE III. Metrotropic Activity of C-21 Haloprogestins in McGinty (Intrauterine) Assay.

Progesterone substitutions			Dose (μg)	No. of animals	Degree glandular development	Relative activity
C-6	C-17	C-21				
			.5	9	2.4*	1
		F	.1	6	2.3	5
		Cl	10.	4	2.0	.05
	OH		100.	4	1.0	Inactive
	"	F	100.	4	1.7	"
	"	Cl	100.	4	1.	"
	OAc		.5	11	2.1	1
	"	F	.5	8	2.2	1
	"	Cl	.05	6	2.1	10
	"	Br	.1	6	2.6	1–5
	"	I	1.	4	3.4	.5–1.†
CH_3			.1	6	2.4	5 †
"	OAc		.02	13	3.	25
"	"	F	.01	12	2.9	50
"	"	Cl	.05	8	2.	10

* McPhail scale. † Preliminary data.

fluoro-acetoxy-progesterone does not fit into the expected potency pattern observed elsewhere.

Summary. The metrotropic activity of a series of C-21 haloprogestins was assayed in the Clauberg, uterine carbonic anhydrase, and intrauterine (McGinty) assays. C-21 Fluorination of both 17α-acetoxy-progesterone and 6α-methyl-17α-acetoxyprogesterone resulted in a marked increase in oral potency of both substances; subcutaneously, potency was unaltered by this structural modification. Upon local instillation of these 21-halogenated progesterone derivatives, decreased potency was observed as atomic weight of substituted halogen increased. 21-Fluoro-17-acetoxyprogesterone failed to increase progestational potency in the intrauterine assay thus differing from other 21-fluoroprogesterones. The most potent metrotropic agent tested was 6α-methyl-17α-acetoxy-21-fluoroprogesterone. Orally, it was twice as potent as its non-fluorinated analogue and 10 times more potent than subcutaneously administered progesterone. It was 50 times more potent than progesterone when both were given subcutaneously and about 50 times more potent than progesterone when given locally.

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Influence of Biotin on Protein Formation in 2 Lactobacilli. (25060)

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The role which biotin plays in metabolism has been investigated extensively. In some instances participation of vitamin in specific chemical transformations has been well established. It has been shown that a deficiency of biotin in the diet of the rat results in decrease in rate of fatty acid synthesis(1). It has been postulated that in the mechanism of this process biotin functions in carboxylation of acetyl-coenzyme A to form malonyl-coenzyme A. A biotin deficiency in chicks has been reported to result in sharp decrease in tissue amylase activity and in albumin synthesis(2). These effects were demonstrated closely related to interference in some of the various reactions involved in the Krebs Cycle in which biotin is known to take part rather than to protein formation as such. Konikova *et al.*(3) reported that excess biotin ingested by biotin-deficient rats resulted in increased protein formation. A more recent investigation by these authors(4) led them to conclude that biotin increased rate of S^{35} methionine incorporation into body proteins. In this connection it seemed worthwhile to ascertain whether or not biotin functioned similarly in growth of certain microorganisms.

Methods. The studies were carried out in an attempt to relate biotin concentration in media to protein formation in cells of 2 microorganisms (*L. casei* and *L. arabinosus*). Cultures of the 2 microorganisms were grown in presence of S^{35} labeled methionine added to a Difco assay medium. Biotin was incorporated in this medium in varying concentrations ranging from 0.00004 μg to 0.016 μg/ml, the lowest level being minimum concentration of vitamin required to support cell growth. Inoculated cultures were incubated at 37°C and growth of the organisms terminated during the log phase. Yield of cells from each condition of growth was harvested, and the cells washed and dried. Total nitrogen/unit weight of dry cells was determined by micro-Kjeldahl procedure and the S^{35} count of hydrolyzed cells measured by automatic scaler.

Results. Typical results are presented in Table I. It appears that biotin incorporated in the medium in excess of that required for optimal growth of the 2 organisms did not result in an increase in nitrogen content or in radioactivity of the harvested cells. In other words, our data do not indicate an increase in protein formation with increased biotin concentration in the medium. While the 2 microorganisms employed responded somewhat differently with respect to range of biotin concentrations required to sustain optimal growth, their general performance was similar. Because of this similarity in response, only typical data for *L. casei* are presented. Nitrogen concentration/unit weight of dry washed cells decreased within the range of 400-fold increase in biotin concentration as indicated by increase in ratio of non-nitrogenous material, whereas radioactivity/unit weight of dry cells remained essentially constant. Results of studies with *L. arabinosus* showed essentially the same trends with somewhat less increase in non-nitrogenous material to nitrogenous material ratio.

TABLE I. Influence of Biotin Concentration in Medium on Ratio of Non-Nitrogenous to Nitrogenous Materials (Non-N/N) and on Ratio of S^{35} Activity (Counts/Min.) to Nitrogen Content (S^{35}/N) of *L. casei* Cells Grown Thereon.

Biotin conc. (μg/ml medium)	Non-nitrogen-nitrogen ratio	S^{35}/nitrogen ratio
.00004	10.8	700
.00008	11.4	660
.00016	12.1	690
.0016	13.1	650
.016	12.9	700

Summary. The data obtained do not indicate that biotin concentration of the medium in excess of that required for optimum growth of organism exerts any positive effect on protein formation in either *L. casei* or *L. arabinosus*. On the contrary, a slight negative effect was noted, particularly when *L. casei* was used as test organism. The results appear to support the observations that lipide formation is preferentially affected by biotin concentration.

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Analyses of a Toxic Factor, Lethal to Paramecium Present in Non-Glass-Distilled Water. (25061)

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While working on the effect of proteolytic enzymes on *Paramecium aurelia*(1) we became aware of an unknown substance in our distilled water which caused the death of this protozoan within 20-30 minutes. The same water, after redistillation in Pyrex glass (to be referred to as doubly glass-distilled), was no longer toxic for these organisms. The toxicity pattern is as follows: First, the ciliate's locomotion is affected, causing it to move erratically in all directions and to spin around on its long axis. Body deformation then sets in usually accompanied by ectoplasmic extrusion, or "blistering" (Fig. 1). Finally, the afflicted organism slows down and succumbs. After this, some degree of disintegration of the cytoplasm of the dead organism occurs (Fig. 2) seldom involving the nucleus. According to Heilbrunn(2) toxic action of distilled water can be attributed in certain instances to presence of poisonous impurities which may presumably represent contamination products from the conventional types of metallic distillation apparatus. It is also known (*loc. cit.*) that a trace of calcium is capable of neutralizing the action of these substances. But the toxic agent or agents were not identified, nor was the nature of their action sufficiently elucidated. The purpose of this study was to use *Paramecium* as the test organism in an attempt to analyze the toxic factor or factors in our distilled water.

Materials and methods. A kappaless stock of *Paramecium aurelia*† was cultured in a medium containing boiled oats. The culture reached peak growth in about 2 weeks, stayed luxuriant for a few more days, then rapidly declined. Doubly glass-distilled water‡ was used exclusively for culturing, making solutions as well as washing glassware. The experimental procedure was as follows: The Paramecia were transferred with a micropipette from an actively growing culture at its peak growth to a depression slide (3″ x 1¾″) with a central 1⅜″ diam. depression. Two drops (40 drops equal approximately 1 ml) of culture medium, containing no less than 100 paramecia, were added to 1½ ml of the test solution. Observations were made at room temperature with both stereoscopic and compound microscopes. A moisture-saturated Petri dish was utilized for maintenance of test cultures for lengthy periods of observation. Stock solutions (0.001 M) were prepared from which appropriate dilutions or combinations were made. Routine pH determinations were made with a Beckman pH meter on all test solutions. 35 mm Kodachrome slides were made with the aid of an AO Spencer 20X dark phase contrast objective. The 3 slides used as illustrations were printed from negatives taken from the original Kodachrome transparencies. A threshold concentration is specified as one which most closely duplicates the toxicity of the distilled water in question, *i.e.*, death of all or nearly all paramecia in 20-30 minutes, preceded by morphological

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† Stock No. 51, courtesy of Dr. T. M. Sonneborn.

‡ Courtesy of Dept. of Biochemistry, Stritch School of Medicine.

changes comparable to those described above

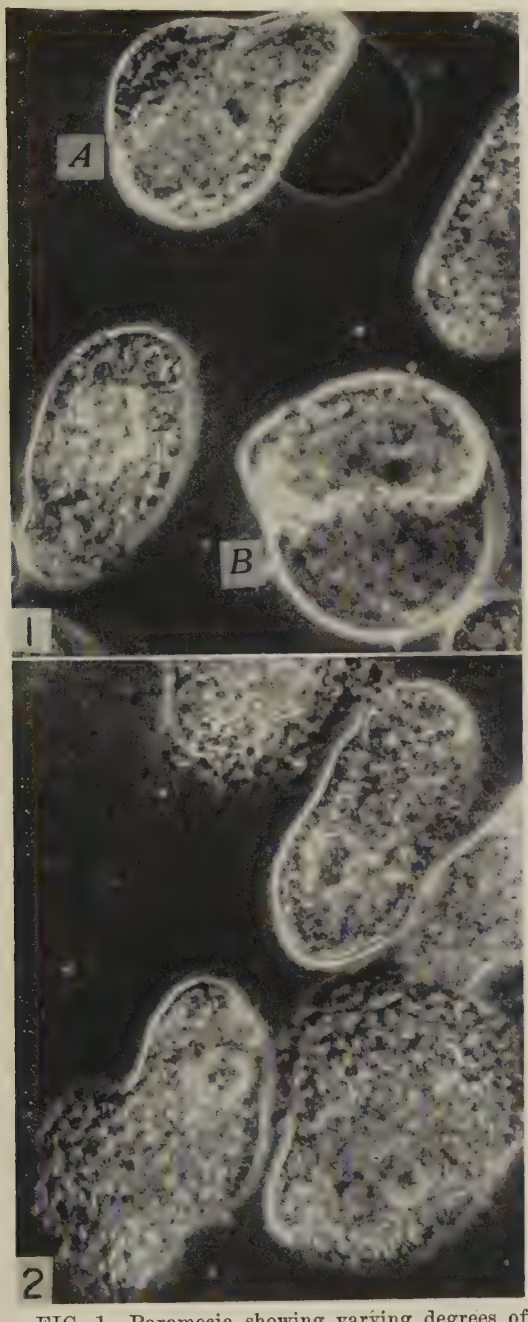

FIG. 1. Paramecia showing varying degrees of body deformation after 16 min. in "toxic" distilled water. Note: 1) typical ectoplasmic "blister" (A); 2) a similar blister filled with endoplasmic granules as a result of pre-mature bursting of plasma membrane (B). Magnification: 250×.

FIG. 2. Dead paramecia undergoing varying degrees of disintegration after 22 min. in "toxic" distilled water. Note intact nuclei in each. Magnification: 250×.

(cf. Fig. 3 with Fig. 1 and 2). It is not a minimum lethal concentration as may be normally denoted by the term "threshold." The reason for this is clear when the results, especially those in connection with the capability of calcium to neutralize the toxic action, are considered. The results presented below represent reproducible observations through repeated trials for each experiment. Three series of experiments were carried out: (a) to determine if change in pH is a factor in the observed toxicity; (b) to duplicate the toxicity of our distilled water by trace quantities of several salts of heavy metals; and (c) to ascertain the role of calcium in counteracting or neutralizing the toxic action of distilled water and salt solutions.

Results. Role of pH. The pH of our distilled water was 5.6, with a normal range of 5.4 to 6.0. After neutralizing this distilled water with $LiCO_3$ or $NaHCO_3$ to pH 7.0, there was no change in its toxicity for *Paramecium aurelia.* To indicate whether $LiCO_3$ and $NaHCO_3$ themselves were toxic to *Paramecium,* each of these salts was

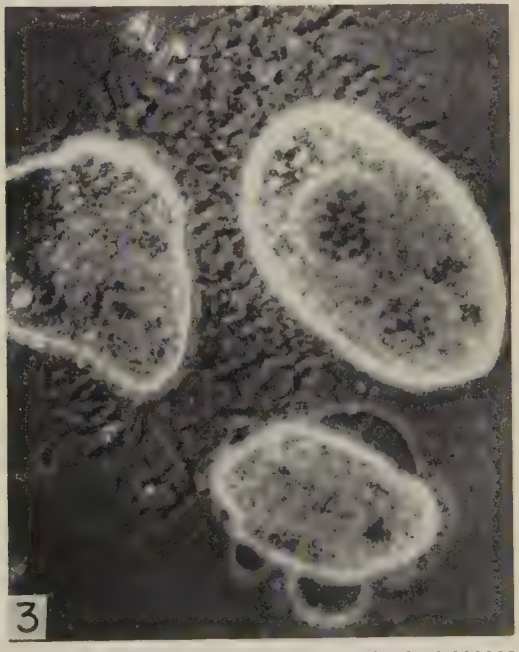

FIG. 3. Three paramecia after 10 min. in 0.000002 M $CuSO_4$ revealing similar toxic symptoms to those caused by the distilled water (one of them showing 2 macro-nuclei, possibly in late anaphase or early telophase). Magnification: 250×.

TABLE I. Threshold Concentrations (M) of 5 Salts Comparable to Toxicity Level Found in Non-Glass-Distilled Water and Lethal to *Paramecium aurelia* in 20-30 Minutes. The chemicals are listed in order of their relative degree of toxicity.

Chemicals	Threshold
$CuCl_2 \cdot 2H_2O$	10^{-6}
$CuSO_4 \cdot 5H_2O$	20^{-6}
$Cu(NO_3)_2 \cdot 3H_2O$	20^{-6}
$Zn(C_2H_3O_2)_2$	10^{-4}
$Pb(C_2H_3O_2)_2$	25^{-4}

dissolved in doubly glass-distilled water in amounts sufficient to bring the pH to 8.9. In both instances no deleterious effect on this organism was observed. These observations coupled with the fact that the small amounts of the salts employed in the experiments brought negligible alteration, if any, to the pH of the distilled water provide sufficient evidence that pH is not a factor in the toxicity phenomenon under consideration.

Duplication of distilled water toxicity by solutions of copper, zinc and lead salts. Solutions of copper chloride, copper sulfate and copper nitrate, and zinc and lead acetate were tested. Observations were made on the toxicity effects of each of these solutions beginning with concentrations of 0.001 M and decreasing to a level at which toxicity symptoms approximated those obtained with the distilled water. The results are summarized in Table I. Approximately 1/100 the threshold concentration rendered these solutions non-toxic.

Role of $CaCl_2$ in neutralizing toxic factor. It was found necessary to determine first the concentration at which $CaCl_2$ itself is toxic to *Paramecium*. A concentration of 0.05 M $CaCl_2$ was lethal, causing death in 10 minutes with explosion of trichocysts. 0.035 M $CaCl_2$ approximated the toxicity level of our distilled water, while 0.02 M proved not only non-toxic by itself, but capable of neutralizing the toxicity of the distilled water. Paramecia lived in this "calciumized" water as well as they did in doubly glass-distilled water. The protection so rendered by calcium varies directly with concentrations of this salt. For instance, with 0.01 M $CaCl_2$ protection lasted only a few hours as compared to 2-3 days with a concentration of 0.015 M. Thus, there seems to be a quantitative relationship between concentration of $CaCl_2$ and extent of protection obtained. Next, we attempted to neutralize by means of $CaCl_2$ the toxicity of the aforementioned salts at their threshold concentrations (Table I). The results reveal a significance of both a quantitative and qualitative nature. Complete neutralization of the toxicity of 0.000001 M cupric chloride (threshold) was obtained with 0.015 M $CaCl_2$. We found this concentration of $CaCl_2$ to be critical, for either lowering or raising it modified the results sharply. For example, within the 0.006-0.013 M range paramecia lived up to a few hours but the presence of 0.017-0.025 M $CaCl_2$ in 0.000001 M $CuCl_2$ caused a slight enhancement of toxicity as evidenced by the fact that death of the organisms occurred sooner here than in 0.000001 M $CuCl_2$ alone. The threshold concentration of lead acetate, 0.0025 M, can be likewise completely neutralized with 0.015 M $CaCl_2$.

In sharp contrast to these results, none of the remaining 3 toxic salts, cupric sulfate, cupric nitrate and copper acetate, was found to be neutralizable by $CaCl_2$. The presence of $CaCl_2$ above 0.013 made these copper salts even more toxic to paramecia, confirming a similar phenomenon with $CuCl_2$ mentioned above. It was imperative to determine whether or not 0.015 M $CaCl_2$, while unable to neutralize the sulfate, nitrate or acetate of copper at their threshold concentrations, might nevertheless neutralize these compounds at lower concentrations. At 1/20 the threshold concentration of both cupric sulfate and cupric nitrate and 1/5 the threshold concentration of zinc acetate, the calcium salt still brought no neutralization. At these sub-threshold concentrations, toxicity symptoms were much milder, and death of the organisms came after many hours of exposure. Also the paramecia were little affected in their movement, and showed less "blistering" and body deformation, as well as no bursting or disintegration of the dead organisms.

An analysis of the reasons for the contrasting behavior of cupric chloride and cupric sulfate toward $CaCl_2$ was accomplished by introducing equal molar strength of NaCl to the cupric sulfate and reciprocally Na_2SO_4 to the cupric chloride, assuming that all 4 salts are

about equally ionized. Addition of $CaCl_2$ to the 2 now ionicly equalized solutions should constitute a crucial test. The results were unequivocal: when $CaCl_2$ of the range 0.013-0.025 M was added to either 0.000001 M $CuCl_2$ + 0.000001 M Na_2SO_4 or 0.000002 M $CuSO_4$ + 0.000002 M NaCl, no neutralization of toxicity due to the copper salts was obtained. This is evidence that the sulfate radical (SO_4^{--}) so introduced has destroyed the power or capacity of $CaCl_2$ to neutralize the toxic action of the otherwise neutralizable $CuCl_2$.

Discussion. This work has demonstrated the toxicity of several metallic salts to *Paramecium.* It is reasonable to assume that others not employed in our experiments may be similarly toxic. Since a comparable pattern of toxicity is revealed by the protozoan under their action as well as that of our distilled water, a general mechanism may be involved in causing death of the organism. Our observations indicate that the ions cause an osmotic change, thereby injuring the plasma membrane in every case. On the other hand, specificity of the different ions is clearly shown by the fact that the different salts required different concentrations to duplicate the toxicity level of the distilled water.

The results have revealed 2 most significant observations: (a) that calcium chloride can neutralize the toxicity of distilled water, cupric chloride and lead acetate, but not the other 3 salts tested, and (b) that SO_4^{--} ions, if present together with calcium, destroys the latter's capacity to neutralize. These findings jointly led us to conclude that the unknown factor in question comprises possibly Cu^{++} and/or Pb^{++} and Cl^- ions. This conclusion is strengthened by the recent work of Bermes (3), who found that traces of the copper ion brought about oxidative changes in isolated human serum lipoproteins. Our results also substantiate previous reports on protective action of calcium against various deleterious effects to aquatic organisms, *e.g.,* marine fish, annelides, and flatworms(4,5,6,7,8).

There are indications that the anions in our experiments also exercise some toxicity to *Paramecium.* Assuming SO_4^{--} and Cl^- to be both mildly toxic, the fact that $CuCl_2$ is twice as toxic as $CuSO_4$ shows that Cl^- is the more toxic of the two. On this basis we may understand why complete neutralization of $CuCl_2$, for instance, was achieved by $CaCl_2$ at a concentration considerably below the latter's threshold toxic level, and that employment of amounts of calcium over this level invariably resulted in an enhanced instead of reduced toxicity. Evidently, in both these instances toxicity of the Cl^- ion plays a role.

Summary. A toxic factor present in our distilled water found lethal to *Paramecium* was analyzed. The results show that it is not related to pH, can be duplicated by 5 metallic salts at specific concentrations (M) as follows: cupric chloride, 10^{-6}; cupric sulfate and cupric nitrate, 20^{-6}; zinc acetate, 10^{-4}; lead acetate, 25^{-4}; and can be neutralized by 0.02 M $CaCl_2$. Calcium chloride is likewise capable of neutralizing cupric chloride and lead acetate, but not cupric sulfate, cupric nitrate or zinc acetate. These results coupled with the demonstration that the presence of SO_4^{--} ions actually destroys the neutralizing power of calcium indicate strongly that the toxic factor under consideration could possibly be copper chloride and/or lead chloride, not likely sulfate or nitrate of copper or zinc acetate. Generally the results also confirm the protective action of Ca^{++} against ionic imbalance or unknown impurities in water as reported by other workers. Because of the demonstrated sensitivity of *Paramecium* to metallic ions we commend its use as an indicator for low-level toxicity due to such ions in solutions including distilled water not made and stored in pyrex glass.

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Studies on Human Leukemia.* (25062)

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Studies on relationship of viruses to origin of tumors have led to important discoveries through employment of methods such as use of newborn animals, electron microscopy of ultrathin sections of different tumors, and tissue culture(1-13). Results of our studies on mouse leukemia and chicken leukosis, based on electron microscopy alone or combined with biological and biophysical methods, have been reported(14-19). In view of results obtained by others(20-23) and ourselves, a study of the relationship of viruses to origin of human leukemia was undertaken.

Material and methods. Lymph nodes obtained by surgical biopsy from patients with different types of leukemia, lymphoma and from patients with non-neoplastic diseases were used. Part of material was immediately fixed for electron microscopy(14); part used for tissue culture; part for histology; and the rest, finely minced and resuspended in 5.3% glucose for storage at $-70°C$. Material for tissue culture was minced, trypsinized in 0.2% trypsin ("Difco" in Ca- and Mg-free Hanks B.S.S.) for 20 minutes at room temperature, centrifuged for 10 minutes at $1,200 \times g$ at 0°C and resuspended in complete medium. Eagle's basal medium with 20% inactivated human serum and 100 units/ml penicillin and 100 μg/ml streptomycin was used. Resuspended cells were grown in T-30 culture flasks. Medium changes were made not less than once a week. Recently, somewhat better results have been obtained using M199 with amino acid mixture, vitamins and glutamine (as used in Eagle's medium), 0.5% lactalbumin hydrolysate (General Biochemicals) and 15-20% calf serum (Colorado Serum Co., Denver). For phase-contrast and electron microscope studies, part of successfully grown material was maintained in Sykes-Moore tissue culture chambers(24). For electron microscopy, cells were fixed 20 minutes in buffered 1% osmium tetroxide, dehydrated and embedded in n-butyl methacrylate using similar time intervals. An RCA EMU 3A electron microscope during earlier and an EMU 3E model during later part of studies were used. Cell-free extracts of biopsy material were tested on monkey kidney cell cultures. Both original and tissue culture material were inoculated into newborn Swiss (Holtzman), C_3HZb/Bi, and C_3H/Bi mice.

Results. Electron microscopy of ultrathin sections of biopsy material revealed changes in cytoplasmic constituents of cells of leukemic and non-leukemic lymph nodes, similar to those observed in cells of mouse leukemia and chicken leukosis(14-19). In addition, characteristic virus particles have been found in cells from lymph nodes of certain cases of leukemia (Table I). No virus particles could be found in several hundred sections of lymph nodes from 5 non-leukemic patients. The particles are present in inclusion bodies (Fig. 1 and 1a) and scattered outside the cytoplasm (Fig. 2). They have an average diameter of 900 Å, an internal dense core, surrounded by one or 2 limiting membranes. There appears

TABLE I. Electron Microscope Study of Lymph Nodes from Biopsy Cases of Human Leukemia.

Diagnosis	Age	Sex	Virus-like particles observed
Acute lymphatic	15	♀	Yes
	28	♂	"
	18	♀	No
	45	♂	"
	22	♀	"
	19	♂	Yes
Chronic monocytic	71	♂	No
Acute monocytic	20	♀	"
Acute myeloid	22	♀	Yes
Lymphosarcoma	48	♂	"
	62	♂	No
Hodgkin's disease	23	♂	"

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to be no difference in size or internal structure of particles in lymph nodes of different types of leukemia and lymphosarcoma.

In cases of acute lymphatic, myeloid leukemia, and lymphosarcoma, in which virus particles were observed, an occasional isolated area in the enlarged lymph node showed presence of these particles in approximately one out of 30 sections. In other areas of this same tissue, no particles were found in several hundred sections examined.

Cells grown *in vitro* from lymph nodes of a number of patients with different types of leukemia and lymphosarcoma revealed changes suggestive of a cytopathogenic agent. Increased granularity of cytoplasm, vacuolization, formation of inclusions, leading occasionally to scattered breakdown of cells, were frequently observed (Fig. 3, 4, and 5). The number of cases examined and results following varying number of passages are shown in Table II. Similar studies of 4 non-leukemic lymph nodes failed to reveal any changes.

Electron microscopy of cells grown in tissue culture from a case of malignant lymphoma (lymphosarcoma) which showed the described changes *in vitro* revealed virus particles in inclusion bodies similar to those seen in ultrathin sections of biopsy specimens (Fig. 6 and 6a).

Cell-free extracts from lymph nodes of 8 patients (2 acute lymphatic, 1 chronic lymphatic, 2 acute myeloid leukemia and 3 malignant lymphoma) have been tested on monkey kidney cell cultures up to 10 sequential passages. Changes, suggestive of a cytopathogenic effect, occurred in all cases of tested leukemic lymph nodes from passages 2 through 8, but were not observed in 9th or 10th passage. Material from tissue culture passages has been tested in mice and part

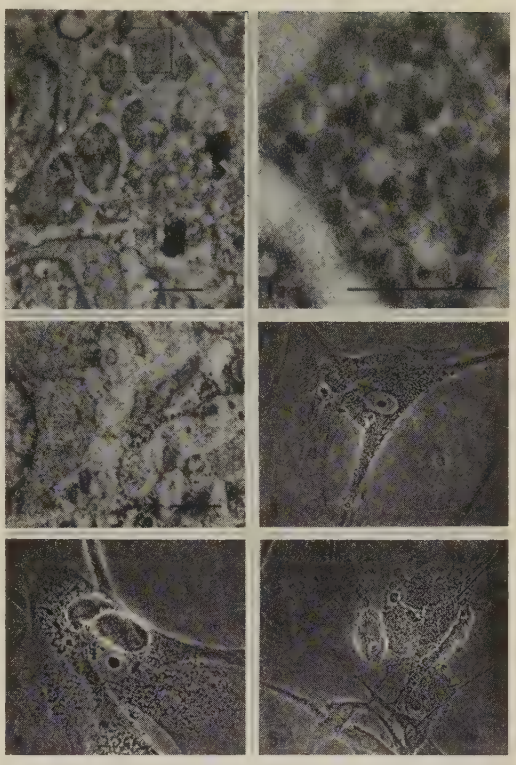

FIG. 1. Electron micrograph of section of biopsy specimen from lymph node of case of lymphosarcoma. Inclusion bodies with virus particles present in cytoplasm of cell. Magnification 6480 ×.

FIG. 1a. Inclusion body with virus particles at higher magnification. 38,880 ×.

FIG. 2. Extracellular virus particles in specimen shown in Fig. 1. Magnification 6480 ×.

FIG. 3. Phase photomicrograph of cell from case of acute monocytic leukemia, 7th passage, 22-day S-M chamber culture, showing perinuclear inclusion. Magnification 108 ×.

FIG. 4. Phase photomicrograph of cells from case of acute myeloid leukemia, 7th passage, 20-day S-M chamber culture, showing cytoplasmic inclusions. Magnification 135 ×.

FIG. 5. Breakdown cells from same case as Fig. 4, 7th passage, 7-day S-M chamber culture. Magnification 108 ×.

TABLE II. Behavior of Human Leukemic and Lymphomatous Tissues *In Vitro*.

Type of tumor	No. grown *in vitro*	Cytopathogenic changes: No. with changes	Cytopathogenic changes: No. of passage	Cytopathogenic changes: Appearance in weeks
Acute lymphocytic leukemia	7	4	2 : 10 : 4 : 1	2– 4
" granulocytic "	4	3	4 : 4 : 6	4– 6
" monocytic "	1	—	—	—
Chronic lymphatic "	2	1	8	12
Hodgkin's disease	2	2	1 : 1	2– 4
Lymphoma excluding Hodgkin's	8	5	1 : 1 : 1 : 2 : 4	2–12
Normal lymph nodes	4	—	—	—

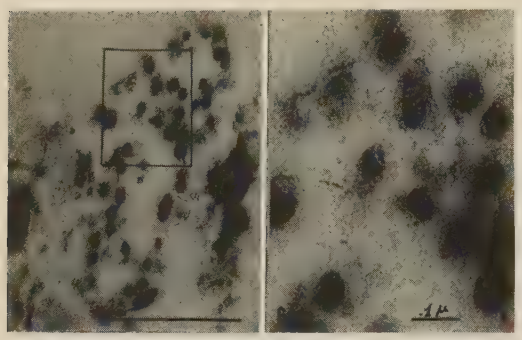

FIG. 6. Electron micrograph of section of cells grown *in vitro*, from lymph node of same case as Fig. 1, 7th passage, showing virus particles. Magnification 18,240 ×.

FIG. 6a. Virus particles at higher magnification in area shown in Fig. 6. Magnification 59,280 ×.

stored at −70°C for future use. Cell-free extract from a non-leukemic lymph node induced no changes during 5 sequential passages on monkey kidney cell cultures.

Results of bioassays of cell-free extracts of biopsy specimens and of fluids from monkey kidney cultures treated with these extracts in newborn Swiss (Holtzman), C_3HZb/Bi and C_3H/Bi mice are shown in Tables III, IV, and V. The incidence of spontaneous leukemia in Swiss (Holtzman) mice is approximately 1% at 12 months of age, and less than 0.5% at much later age in C_3HZb/Bi and C_3H/Bi mice. The induction up to 18% of leukemia at an early age by material from one case of acute lymphatic leukemia cannot be considered significant, in view of the development of leukemia in 10% of uninoculated litter-mate control animals at a similar age. In addition, newborn Swiss (Holtzman) mice injected with untreated monkey kidney culture fluids developed increased incidence of leukemia (Table VI). The incidence of mammary tumors (MT) and hemorrhagic disease (H) in inoculated animals was comparable to that in uninoculated litter-mate controls.

Discussion. The study of ultrathin sections of lymph nodes from 12 patients with leukemia or malignant lymphoma revealed in cells of all cases, vacuolization of cytoplasm, breakdown of mitochondria and endoplasmic reticulum, which were also found in cells of lymph nodes from 5 patients with non-neoplastic disease. In addition to these changes, inclusion bodies with spheroidal particles of a characteristic structure were found in the cytoplasm of cells from lymph nodes of 3 patients with acute lymphocytic, 1 with acute myeloid leukemia, and 1 with lymphosarcoma. The virus particles are in all cases of similar size and appearance. They have not been observed in cells of non-leukemic lymph nodes. The particles may represent a virus unconnected with leukemia.

Changes suggestive of presence of a virus have been observed in cells of lymph nodes grown *in vitro* from 15 out of 24 cases with different types of leukemia and lymphosarcoma. Such changes have not been seen in cells of lymph nodes during a similar number of passages in tissue culture from 5 patients with non-neoplastic disease. They have been

TABLE III. Newborn Mice Injected with Extracts of Human Leukemic Organs.

Exp.	Diagnosis	Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia development (mo)
HL #1	Acute lymphocytic leukemia	15–17	Swiss —Inj.	44/244 (+2H)	9
			—Controls	6/65	10
			C_3HZb—Inj.	3/50 (+1MT) (+1H)	10
			—Controls	0/6	—
HL #2	Acute myeloid leukemia	15	Swiss —Inj.	3/15	8
			—Controls	1/7	14
			C_3HZb—Inj.	1/14 (+1H)	5
			—Controls	0/11	—
HL #3	Acute myeloid leukemia	12	Swiss —Inj.	1/23 (+2H) (+2MT)	5
			—Controls	1/9	8
			C_3HZb—Inj.	0/6	—
			—Controls	0/5	—

TABLE IV. Newborn Mice Injected with Extracts of Human Leukemic Organs.

Exp.	Diagnosis	Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia development (mo)
HL #5	Lymphosarcoma (follicular lymphoma)*	11	Swiss —Inj.	5/72 (+3MT) (+1H)	5
			—Controls	2/34	7
			C_3HZb—Inj.	1/25	6
			—Controls	0/13	—
HL #6	Lymphosarcoma (lymphocytic type)	10	Swiss —Inj.	5/54	5
			—Controls	2/32	8
			C_3HZb—Inj.	0/28	—
			—Controls	1/16	8
HL #8	Chronic lymphocytic leukemia	8	Swiss —Inj.	1/30	6
			—Controls	1/20	5
			C_3HZb—Inj.	1/28	6
			—Controls	0/14	—

* And chronic lymphocytic leukemia.

TABLE V. Newborn Mice Injected with Fluids from Tissue Cultures Treated with Extracts of Human Leukemic Organs.

Exp.	Diagnosis	Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia development (mo)
HL #5	Lymphosarcoma (follicular lymphoma)	11	Swiss —Inj.	3/60	7
			—Controls	3/40	5
			C_3H —Inj.	1/40	4
			—Controls	0/18	—
HL #6	Lymphosarcoma (lymphocytic type)	10	Swiss —Inj.	12/177 (+3MT)	6
			—Controls	5/92 (+3MT)	8
HL #7	Acute lymphocytic leukemia	9	Swiss —Inj.	12/176 (+2MT)	6
			—Controls	7/96 (+3MT)	8

found in cells of leukemic lymph nodes, both positive and negative for presence of virus particles in electron microscope studies. In one case of lymphosarcoma in which virus particles were seen in the biopsy specimen, similar virus particles were also demonstrated in cells of this material grown *in vitro* which had shown cytopathogenic changes.

Monkey kidney cell cultures, when used for testing extracts of leukemic lymph nodes, present a drawback because of presence of simian viruses. However, control cultures treated with fluids from uninoculated monkey kidney cultures failed to show any changes. Extracts of lymph nodes have also been tested on human embryo cultures. In tests, so far carried out, changes have been observed during first passage which could not be transmitted serially. Experiments to isolate an infective agent in human embryo cultures are being continued using different technics.

In bioassays of cell-free extracts of biopsy material from leukemic lymph nodes and of fluids of monkey kidney cell cultures treated with extracts of these lymph nodes, no significant difference in incidence of leukemia was observed between inoculated and uninoculated litter-mate control animals. The increase in incidence of leukemia in uninoculated littermate controls and in mice, following inoculation of untreated culture material, is of interest. Inhibition of hemagglutination tests (25), using sera of Swiss (Holtzman) mice from our colony against polyoma virus (ob-

TABLE VI. Newborn Mice Injected with Untreated Monkey Kidney Fluids.

Duration of exp. (mo)	Mice used (litter-mates)	No. with leukemia/No. inj.	Avg age of leukemia developm't (mo)
7 to 10	Swiss–inj.	6/104	6
	–controls	5/41	5

tained through the courtesy of Dr. Sarah Stewart), have shown high titers indicating infection with the virus. It should be noted, however, that fluids from cultures of lymph nodes showing cytopathogenic changes failed to give hemagglutination of guinea-pig red cells(26).

Summary. 1) Electron microscope studies have been carried out on ultrathin sections of lymph nodes from 6 patients with acute lymphatic, 1 with acute and 1 with chronic monocytic, 1 with acute myeloid leukemia, 2 with lymphosarcoma and 1 with Hodgkin's disease. Virus particles of approximately 900 Å in diameter have been observed in cells of lymph nodes from 3 cases of acute lymphatic, 1 of acute myeloid leukemia and 1 of lymphosarcoma. Similar studies of sections of lymph nodes from 5 non-leukemic patients have failed to reveal presence of virus particles. 2) Cytopathogenic changes have been observed in cells of lymph nodes grown *in vitro* from 15 out of 24 patients with leukemia and lymphosarcoma. Cells of lymph nodes grown *in vitro* from 4 non-leukemic patients have not shown changes observed in lymph nodes from leukemic patients during similar number of passages. Cell-free extracts of lymph nodes from 8 patients tested have induced changes in monkey kidney cell cultures which could be serially transmitted. These changes have not been seen in similar cultures treated with extract of a non-leukemic lymph node. Extracts of leukemic lymph nodes which induced changes in monkey kidney cell cultures did not have a similar effect in human embryo cultures. 3) Cell-free extracts of biopsy material from leukemic and lymphomatous lymph nodes and fluids from monkey kidney cell cultures treated with these extracts have not shown specific leukemia-inducing activity in Swiss (Holtzman), C_3HZb/Bi and C_3H/Bi strain mice. 4) It is of particular interest that in one patient with lymphosarcoma, not only were virus particles observed in ultrathin sections of the biopsy specimens, but also similar virus particles have been found in cells from the same specimen, grown *in vitro* for 6 sequential passages during which cytopathogenic changes had been observed.

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Renal Tubular Secretion of Magnesium in Dogs.* (25063)

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The mechanism for renal excretion of magnesium has not been determined. Since Bieter(1) demonstrated that magnesium is excreted by the aglomerular fish kidney, the possibility of tubular excretion of magnesium by the mammalian kidney has remained an open question despite several previous studies utilizing conventional clearance technics(2,3,4). Development of the "stop flow" technic for localization of transtubular transport along the nephrons(5,6) has afforded a new approach for study of this problem in animals.

Method. Six female dogs were anesthetized with intravenous pentobarbital in doses not in excess of 30 mg/kg. The right ureter was cannulated with polyethylene tubing which was advanced to just below the junction of ureter and pelvis and tied securely. The other ureter was cannulated and allowed to drain freely. A priming dose of creatinine (0.08 g/kg) was given intravenously over a period of one minute. 20% mannitol in 0.8% sodium chloride and 0.2% creatinine was administered intravenously by means of a constant infusion pump at a rate of 10 ml per minute. An equilibration period of 45 minutes was allowed. The ureteral catheter was clamped for a period of 6 to 8 minutes. Two minutes prior to release of the clamp a solution containing 150 mg para-amino hippurate (PAH), 1 g inulin, and 6.3 to 25.0 μc magnesium 28 (Mg^{28}) (125 to 500 mg $Mg(OH)_2$) was injected over a period of 2 minutes. The ureteral clamp was then released and serial urine samples of approximately 0.6 ml were collected. Volume, radioactivity, PAH, inulin, creatinine, sodium and potassium concentrations and pH were determined on all urine samples and on three 3-minute additional control samples prior to and following the occluded periods. Blood samples were collected just prior to and immediately following the occluded interval for plasma magnesium, creatinine, sodium and potassium concentrations. Creatinine U/P ratios were used to correct for water reabsorption along the nephrons. PAH was determined according to Smith(7), creatinine by the method of Bonsnes and Taussky (8) and inulin according to Schreiner(9). Sodium and potassium were analyzed with a Baird internal standard flame photometer, and radioactivity was counted utilizing a well type scintillation counter. Volume of the serial urine samples was determined by weight.

Results. Fig. 1 illustrates an example typical of the 6 experiments and the drawing at the top of the figure represents interpretation of its significance. Mg^{28} appeared in the fourth urine sample and radioactivity gradually increased until the filtered samples began to appear, whereupon the radioactivity showed a second pronounced rise. Mg^{28} appeared just proximal to the sharp peaks in pH change and in potassium change, 8 samples prior to the appearance of PAH, and 16 samples prior to the appearance of inulin. The variable level of plasma magnesium obtained in our 6 experiments (1.79 to 4.53 meq/l) did not influence the apparent site at which Mg^{28} appeared in the urine samples. All experiments yielded similar results (Table I).

Discussion. Renal magnesium excretion by a secretory process has been demonstrated in the aglomerular fish by the high content of magnesium in the urine(1,10). Whether or not such a process exists in the mammalian kidney has been a more difficult problem, although suggested by Hirshfelder(11) as early as 1934. Previous observations in normal men and in patients with chronic renal disease(2,3) were inconclusive. Experimental observations in dogs with magnesium loading revealed that in some dogs a ratio of slightly above unity (1.1) was attained for magnesium excreted over magnesium filtered when the amount of magnesium filtered was calculated from the ultrafiltrable concentration of mag-

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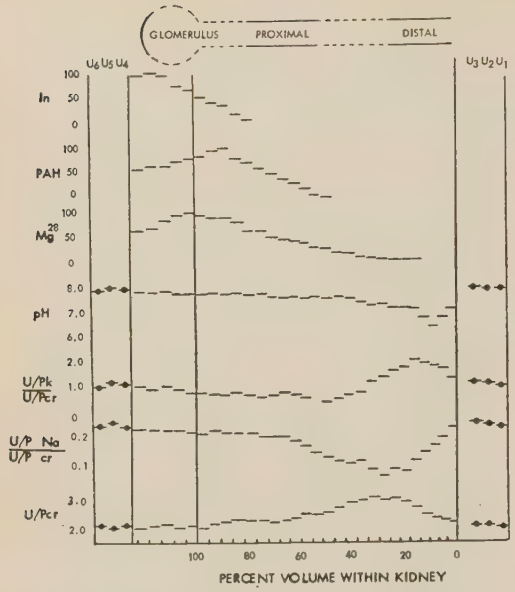

FIG. 1. Results of "stop-flow" analysis using magnesium-28. Magnesium-28 counts, inulin, and PAH are recorded in % of maximum excreted. Urine volume is recorded in % within the kidney using all samples distal to 50% maximal inulin excretion.

nesium in plasma(4). These data suggested, but were inconclusive evidence of, renal tubular secretion of magnesium. Recently Robinson *et al.*(12) have also stated that nonfiltered Mg^{28} can enter the renal tubule.

The pitfalls of the present technic are: nephrons are of variable lengths and have variable transit times; there is necessarily some mixing of fluid from the various nephrons at site of the renal pelvis; the nephrons are subjected to a highly abnormal, marked osmotic diuresis; the column of fluid is acted upon under a static condition; and, the more proximal fluid must pass through the more distal tubule and thereby has been acted upon by the more distal tubular epithelium. Nevertheless, the samples follow an orderly and characteristic pattern resulting in an informative degree of localization of tubular function.

TABLE I. Results Obtained in 6 Dogs Using "Stop-Flow" Analysis and Magnesium-28. Values given represent % level in nephron where the various substances appear.

Dog	Magnesium-28	PAH	Inulin
1	20	56	79
2	17	55	77
3	19	57	72
4	16	47	74
5	19	52	75
6	14	47	76
Mean & S.D.	17.50 ± 2.06	52.33 ± 4.11	75.50 ± 2.22

We consistently found that Mg^{28} appeared in the tubular urine distal to excretion of inulin and PAH. This is strongly suggestive that magnesium is secreted by the distal tubule.

Summary. Excretion of magnesium has been studied in dogs utilizing the "stop-flow" technic and Mg^{28}. The evidence indicates that magnesium is secreted by the distal tubule.

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Reversal of Cycloserine Inhibition by *D*-alanine.* (25064)

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Bondi *et al.*(1) showed that the inhibitory action of cycloserine (*D*-4-amino-3-isoxazolidone)(2,3) on several strains of *Staphylococcus aureus* is competitively antagonized by *D*- or *L*-alanine. According to findings of Ciak and Hahn(4) natural *D*-cycloserine can cause protoplast formation in *Escherichia coli* and accumulation of N-acylamino sugar in *Staph. aureus,* while the synthetic *L*-isomer does not produce these effects. These authors have related the similarity of action of *D*-cycloserine and penicillin on bacterial cell wall formation to their similarity in chemical structure. Park (5) has suggested that cycloserine might "prevent normal incorporation of *D*-alanine into the wall." During studies with *Streptococcus faecalis,* we found that the inhibitory action of natural cycloserine on cells that are not actively growing but are making cell wall substance(6) is specifically reversed by the *D*-form but not by the *L*-form of alanine.

Methods. Growth of *S. faecalis* (ATCC 9790) in complete synthetic medium(7) was stopped during logarithmic growth phase by rapid chilling and the cells harvested and thoroughly washed aseptically at 0 to 2°C. Aliquots of cell crop were then placed in "Wall Medium" which contained/ml : *L*-glutamic acid, 0.3 mg; *L*-aspartic acid, 0.1 mg; *L*-lysine, 0.1 mg; *DL*-alanine 0.2 mg; *L*-cystine, 0.2 mg; guanine, 0.03 mg; uracil, 0.03 mg; ammonium sulfate, 0.6 mg; sodium acetate, 1.2 mg; glucose, 10 mg; sodium phosphate buffer, pH 6.5, 0.3 m mole; magnesium sulfate 0.1 mg; ferrous sulfate 5.5 μg; manganous sulfate 6.8 μg. The cells were incubated at 38°C and their optical density read at frequent intervals(8). In later experiments the organism was grown in Vit. B_6 deficient medium (less than 10^{-6} μg/ml measured as pyridoxamine with *S. faecalis*) with one-third of *DL*-alanine concentration (0.2 mg/ml) previously employed. While this amount is sufficient to give logarithmic growth to high turbidities, it significantly reduces amount of intracellular alanine which, in absence of added alanine, is available for cell wall synthesis. Under these conditions a study was made of the effect of penicillin and cycloserine† on cell wall synthesis.

Results. When *S. faecalis* cells are taken from the log phase and incubated in the relatively simple "Wall Medium" a turbidity increase that is accompanied by synthesis of additional cell wall substance takes place(6). When synthesis of additional wall substance is prevented, such as by lack of a nutrient essential to the cell wall, cells taken from log phase are prone to autolysis either in growth medium or in "Wall Medium"(7,8). Fig. 1 shows turbidity increase in "Wall Medium" and the inhibitory effect of several levels of penicillin (Fig. 1a) on this process. In complete growth medium growth of the same quantity of cells will not be inhibited by 33 μg/ml of penicillin. Contrary to the penicillin effect, as much as 200 μg/ml of cycloserine causes little inhibition in complete "Wall Medium" (Fig. 1b, curve 2). When alanine is omitted from this medium the overall response is only slightly depressed (curve 3) but it is now sensitive to cycloserine (curve 4), and in fact, 25 μg/ml of the antibiotic will lead to rapid lysis. Presence of *L*-alanine (curve 4) has no effect while addition of *D*-alanine partially reverses the cycloserine inhibition (curve 5). Further tests indicated that *D*-alanine was effective in far lower concentration than was *L*-isomer (*i.e.*, 1.6 μg/ml of the *D*-form was approximately equivalent to 12.5 μg/ml of the *L*-form in reversing the action of 25 μg/ml of cycloserine). Since Vit. B_6 is involved in synthesis and racemization of alanine, B_6 deficient cells were employed in

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† Cycloserine was supplied by Eli Lilly and Co. (trade name Seromycin).

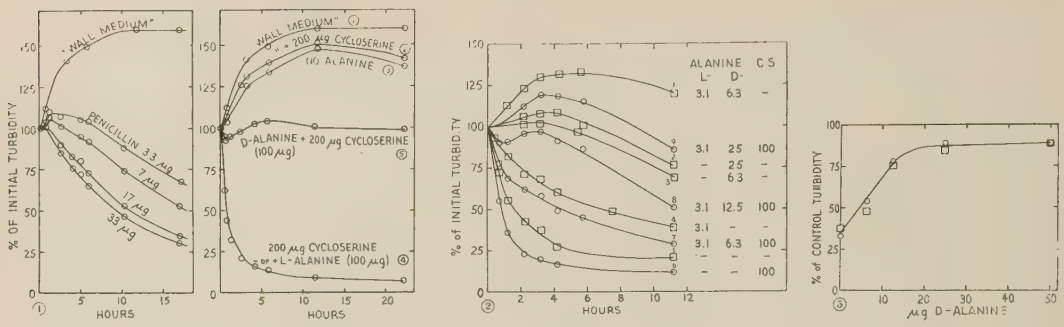

FIG. 1. Effect of penicillin (Fig. 1a) and cycloserine (Fig. 1b) on incubation of log phase *S. faecalis* cells in "Wall Medium." Amounts indicated are μg/ml. Results expressed as % of turbidity of incubated tubes at time 0. *DL*-alanine was omitted from "Wall Medium" for curves 3 to 5 of Fig. 1b, with supplements as indicated.

FIG. 2. Effect of cycloserine on incubation of Vit. B_6 deficient *S. faecalis* cells in "Wall Medium" without *DL*-alanine. Individual alanine isomers were added as indicated. Amounts are μg/ml; CS indicates cycloserine. Curves 1 to 5, with square symbols represent experimental tubes without cycloserine, curves 6 to 9 (circles) with cycloserine (100 μg). Curve 6 also represents 3.1 μg *L*-alanine, 0 μg *D*-alanine, 100 μg CS. Results expressed as in Fig. 1.

FIG. 3. Effect of *L*-alanine concentration on reversal of cycloserine effect by *D*-alanine during incubation in "Wall Medium." Results expressed as % of turbidity attained after 3 hr incubation (Fig. 2) of identical control tubes without cycloserine. Cycloserine concentration, 100 μg/ml. Square symbols represent, per ml, the presence of 3.1 μg *L-alanine*, the circles 50 μg *L*-alanine.

further experiments. B_6 deficient cells require both alanine isomers for wall synthesis (Fig. 2, curves 1 to 5). Curves 6 to 9, Fig. 2, show the effect of increasing concentration of *D*-alanine, in presence of a constant amount of *L*-alanine (3.1 μg/ml), on inhibition produced by 100 μg/ml of cycloserine. Fig. 3 demonstrates that reversal of cycloserine action by *D*-alanine is independent of concentration of *L*-alanine over a wide range (3.1 to 50 μg/ml), and that inhibition is reversed by *D*-alanine at one-fourth the amount of antibiotic by about 90%. *D*-alanine was proportionally effective over the range of cycloserine concentrations tested (25 to 500 μg/ml), indicating a competitive relationship(9).

Discussion. The reversal of cycloserine inhibition seems to be specific for *D*-alanine. It seems probable that the small effect of the *L*-isomer, particularly at high concentrations, is due to a low level of contamination of the product used with *D*-alanine or Vit. B_6 active substances.‡ None of the amino acids of the "Wall Medium" antagonized the action of penicillin, nor was the action of cycloserine antagonized by any of the amino acids except *D*-alanine.

Cycloserine seems to be one of the few examples of an antibiotic that is a logical antagonist. It is a derivative of *D*-serine and a structural analog of a number of neutral *D*-amino acids of which only *D*-alanine has thus far been found to play an active part in bacterial cell synthesis. It would not be surprising if a number of neutral *D*-amino acids were found to act in a manner similar to cycloserine. The action of neutral *D*-amino acids in producing protoplast-like structure of *Alkaligenes faecalis*(11) may be such an example.

The competition of cycloserine and *D*-alanine is not at a permeability site as indicated by the reversing effect of intracellular *D*-alanine. As postulated by other workers(4,5), it may take place at site of incorporation of *D*-alanine into a wall precursor(12). Despite their structural similarity(4,13), and despite the fact that both interfere with the same biosynthetic process, penicillin and cycloserine differ in their mode of action. Their synergistic effect(2) may be due to sequential blocking of cell wall synthesis.

Summary. *D*-alanine, but not *L*-alanine,

‡ Vit. B_6 active substances were found in 5 high quality commercial *L*-alanine products and in 1 *L*-alanine sample obtained from Dr. J. Greenstein in 1951, and these were removed from the *D*- and *L*-alanine used in present experiments by treatment with activated charcoal(10).

competitively reverses the inhibitory action of cycloserine on *S. faecalis* cells that are synthesizing cell wall substance. Both cycloserine and penicillin can inhibit cells that are not growing but that are making cell wall substance. However, they differ in mode of action.

I am indebted to P. Conover for skillful technical assistance and to Dr. G. Toennies for helpful advice.

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Factors Responsible for Decline of Inflammation in Arthus Hypersensitivity Vasculitis.*† (25065)

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In recent studies of Arthus-type vascular inflammatory reactions(1,2), a rough correlation was found to exist between disappearance of antigen from the vessel and a decrease of inflammation in the reaction. This was noted both macroscopically, and in the vessels, microscopically. These results suggested that removal or perhaps some form of isolation of the antigen-antibody complex eliminated the phlogogenic stimulus and allowed healing of the damaged vessels to take place. However, such factors as a possible exhaustion of cellular or humoral components essential to development of the reaction, and/or production of inhibitors locally might account for the decrease in inflammatory reaction also. In order to find if these latter factors played an essential role in the decline of inflammation, experiments were performed in which antigen was reintroduced into healing Arthus sites at various times. This was performed under the supposition that any inflammation secondary to the presence of the reinjected antigen and its corresponding antibody would be prevented or at least decreased in intensity, if an exhaustion of essential materials had occurred, or if inhibitors played an important role.

Materials and methods. Antigen: Bovine serum albumin (BSA), Armour Laboratories, Lot #T-68204 was used to elicit the Arthus reactions. Aside from BSA, other antigens used for control purposes or to obtain antibodies for the fluorescent antibody studies included rabbit serum albumin (RSA) and rabbit serum globulin (RSG), (Fraction II), Pentex, Inc., Lot #6901 and human gamma globulin (HGG), (Fraction II), Squibb Laboratories, Lot #1615. *Antisera*: Antibodies to the antigens mentioned above were produced, characterized and absorbed to remove con-

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§ Public Health Research Fellow of Nat. Inst. of Neurol. Dis. and Blindness.

TABLE I. Macroscopic Reaction to Reinjection of Antigen into Healing Arthus Sites in Rabbits.*

Time of reinj. (after 1st inj.) (hr)	No. of rabbits	Reinj. antigen	Reactions†	
			Non-reinj. site	Reinj. site
48	3	75 μg N BSA	2+, 1+, 2+	4+, 4+, 4+
72	3	*Idem*	±, ±, ±	3+, 4+, 4+
	2	75 μg N HGG	±, ±	+, +
	2	27 μg N BSA	±, ±	3+, 3+
96	3	75 μg "	±, ±, ±	4+, 4+, 4+
216	3	*Idem*	±, ±, ±	4+, 4+, 4+

* Original Arthus reactions produced by I.D. inj. of 75 μg BSA N.
† Reactions read 7 hr after reinjection. Injections into virgin sites closely resembled reinjection sites in each case.

taminating antibodies as described previously (2). *Production of reactions*: Adult male albino rabbits were used for this study. The hair at the reaction site was carefully removed with electric clippers. (a) Active or classical Arthus reactions were produced by injecting 75 μg BSA nitrogen (N) intradermally (I.D.) into rabbits previously sensitized and containing between 150 and 1100 μg anti BSA N/ml serum. Reinjections of the antigen into the healing reactions were performed by injecting 27 or 75 μg BSA N into the identical site of the first injection at various times after the first as will be indicated later. Volumes of injected antigen were 0.15 ml or less. Reactions were graded as previously(1,2), and biopsies were taken either at 7, 16, or 24 hours after the second, superimposed injection. In each case half the biopsy was fixed in 10% formalin for hematoxylin and eosin (H&E) staining, while the other half was rapidly frozen at –70°C for fluorescent antibody studies. *Fluorescent antibody technic*: Frozen tissue sections were studied using the fluorescent antibody technic of Coons and Kaplan(3) with certain modifications(1).

Results. Four Arthus reactions were produced on the flanks of each of 15 sensitized rabbits. Typical severe hemorrhagic, edematous reactions appeared within 4 hours, reaching a maximum between 7 and 10 hours, declining somewhat in intensity at 48 hours and to a much greater extent at 72 hours. Microscopically, the classically described acute vasculitis was present in sections taken at 24 hours and fluorescent antibody studies at this time revealed the presence of antigen localized in the walls of diseased vessels. However, by 48 hours microscopic evidence for healing of the lesions was apparent with loss of most of the polymorphonuclear leukocytes (polymorphs, or rabbit heterophiles) in and around damaged vessels and with the presence of many mononuclear cells and a scattering of eosinophiles. Necrotic material and thromboses were still present at 48 hours although to a lesser extent than 24 hours. By 72 hours further evidence of resolution was present. Fluorescent antibody studies at 64 hours revealed that little or no antigen remained in vessel walls similar to that previously described for 48 hour reactions(2). The occasional small flecks of antigen remaining appeared to be surrounded by mononuclear cells and might well have been isolated from the surrounding milieu by this means.

Reinjection of 75 μg BSA N intradermally into the site of the healing lesions was performed at 48, 72, 96 or 216 hours after the original antigen injection. Three rabbits were used at each time of reinjection. To study the effect of small amounts of antigen, 2 rabbits were reinjected 72 hours after the first injection with 27 μg BSA N. In each rabbit a virgin site was also injected for purposes of comparison. As control, one of the healing sites on each of 2 rabbits was given a similar superimposed injection of 75 μg N HGG at 72 hours. The results are shown in Table I. In each case in which BSA was reinjected, a severe hemorrhagic edematous reaction rapidly ensued identical to not only the original Arthus reactions, but also to the virgin sites made at the time of reinjection (Fig. 1). The reactions to the reinjected antigen also developed at the same time as the virgin sites,

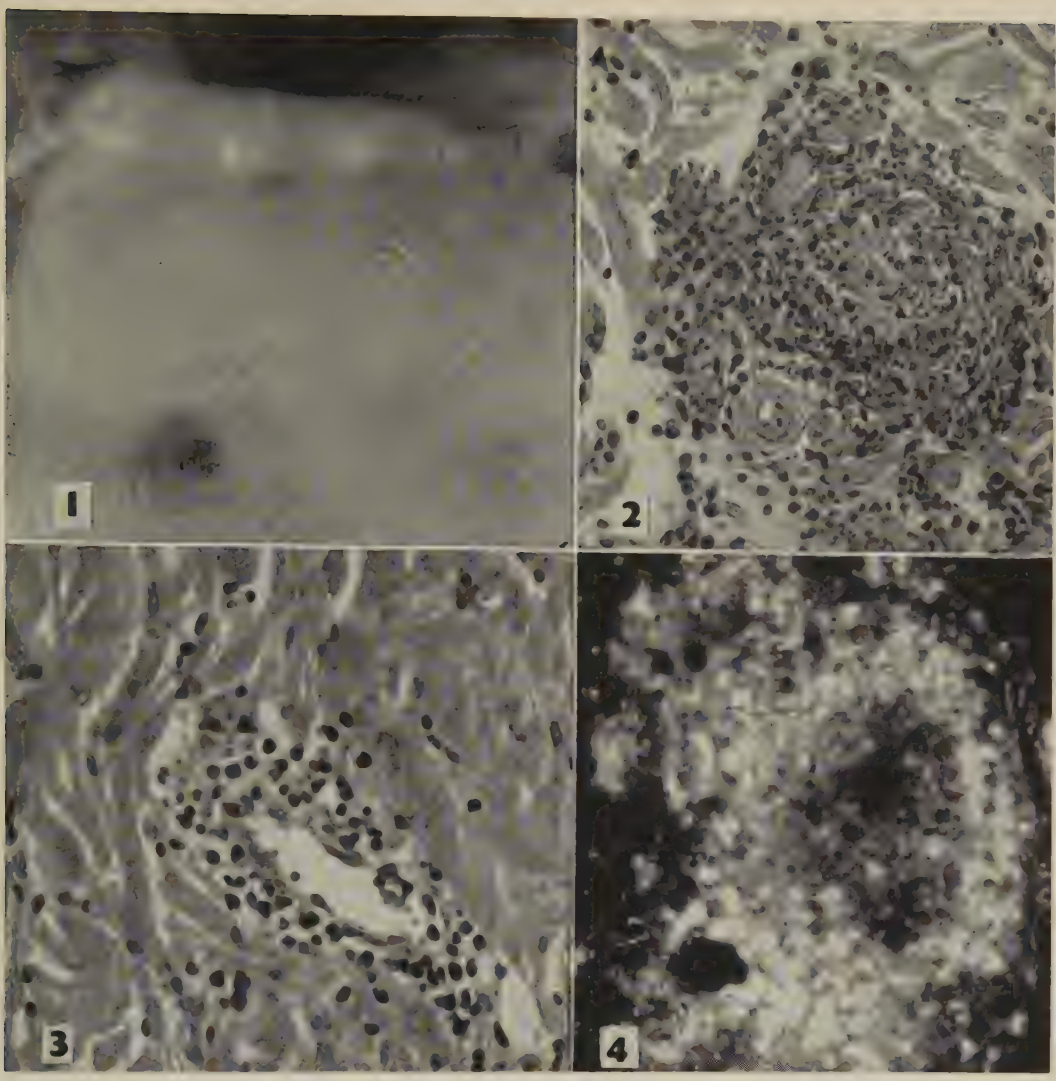

FIG. 1. Photograph of rabbit showing an Arthus reaction produced by a superimposed inj. of 75 μg BSA N into a healing 72-hr-old Arthus site. A 72-hr healing site is seen at right for comparison. Photo taken 6 hr after reinjection. × 0.6.

FIG. 2. Photomicrograph showing acute vascular inflammation 6 hr after reinjection of 75 μg BSA N into a 72 hr Arthus site. Section was taken from a reaction similar to that seen in Fig. 1. Hematoxylin and eosin. × 300.

FIG. 3. Photomicrograph showing a vessel in a section of healing Arthus site at 72 hr. Note that leukocytes are mononuclear in type and little evidence of inflammation persists in contrast to vessel seen in Fig. 2. Hematoxylin and eosin. × 300.

FIG. 4. Fluorescence photomicrograph showing presence of BSA in wall of an inflamed vessel similar to that seen in Fig. 2. Section was taken from a reinjected Arthus reaction 6 hr after second inj. This indicates that after reinjection of antigen in skin, antigen and antibody again concentrate in vessel walls. × 280.

showing distinct evidence of reaction by 2 hours. The sites not reinjected continued the normal resolution in each case showing the normal course of events of a healing Arthus reaction. The control superimposed injections of HGG failed to cause more than a mild edema and erythema that largely disappeared by 6 hours.

Microscopic sections of these lesions taken either at 7, 16, or 24 hours after reinjection of

BSA revealed severe vascular inflammatory changes (Fig. 2). These acutely damaged vessels were found throughout the section. In particular, acutely inflamed vessels were found in areas where mononuclear cells were present in considerable numbers. These sections were similar to the virgin injection sites except that an abundance of mononuclear cells was also present. The healing sites that were not reinjected showed the usual resolution seen at that particular time after antigen injection (Fig. 3).

Fluorescent antibody studies of the reinjected sites again revealed localization of the antigen and RSG in the vessel walls throughout the section (Fig. 4). This suggested that the specific etiologic agents, *i.e.*, antigen and antibody had again combined in the vessel walls.

Controls for the fluorescent antibody studies included the use of unrelated fluorescent sera, *i.e.*, fluorescent anti HGG and fluorescent normal rabbit globulin which yielded negative fluorescence in each case. Fluorescent anti RSA failed to show concentrations of RSA in the vessel wall although albumin was found diffusely scattered in the dermis. As further control, sections were flooded first with *non*-fluorescent anti BSA for 20 minutes and then, after washing, with fluorescent anti-BSA for 10 minutes and the intensity of these slides was compared with slides flooded first with non-fluorescent anti HGG for 20 minutes and then fluorescent anti BSA for 10 minutes. In each case the latter section revealed far greater intensity of fluorescence indicating that the fluorescence marking of the BSA could be blocked by prior application of unlabelled specific antibody.

Discussion. Previous studies have indicated that loss of antigen from a hypersensitivity vasculitis of the Arthus type corresponded in time with a diminution of the vascular inflammatory reaction. The studies reported here would indicate that it is the loss of the offending antigen that is necessary before diminution of reaction and healing of the vessels take place. This is in keeping with the hypothesis that the antigen and antibody are the phlogogenic agents responsible not only for initiation, but also for continuation of the Arthus vascular reaction. This conclusion is supported by the finding that reactivation of vascular inflammation in healing vessels followed the union of fresh antigen and antibody in the vessel walls.

Two other conclusions seem possible: 1). That the various cellular and humoral factors essential to development of the inflammation are present in quantities capable of supporting an inflammatory reaction at a time when the Arthus reaction is subsiding. It would seem, therefore, that any depletion of these essential elements does not play an important role in diminution of the vascular reaction. It was found that reunion of antigen and antibody in the healing vessels after reinjection was again accompanied by an influx of polymorphs, which from previous studies(2,4,5) have been shown to provide an essential key to the reaction. This stresses the important role of polymorphs in development of the vascular inflammation. Rosenberg, Chandler and Fischel(6) have recently found that passive cutaneous anaphylaxis reactions can be elicited in the same skin site in guinea pigs on 3 consecutive days suggesting that in these reactions also the materials necessary to support the inflammation were present in sufficient quantity. Stone(7) has shown that during protracted systemic anaphylaxis in mice and guinea pigs that development of Arthus reactions was inhibited. Bier *et al.*(8) and Osler *et al.*(9) have demonstrated an inhibition of passive cutaneous anaphylaxis in rats following protracted anaphylaxis, and have indicated that depletion of complement is responsible for the inhibition. The lack of inhibition of the reactions in our studies in contrast to that noted by these authors might be explained in several ways: First, in the experiments quoted above, a relatively severe systemic reaction took place, perhaps capable of removing considerably more of the factors essential for development of inflammation than are removed in the local Arthus reactions studied here. Second, the reactions that were apparently inhibited were produced at a relatively short time after or even during the process of exhaustion, while our testing reactions were performed at a much later time. Furthermore, the importance of complement is

apparently not as great in development of Arthus reactions as in passive cutaneous anaphylaxis, and also the ease of depleting an animal of factors essential for inflammation might well vary among different species.

2). The finding reported here would also suggest that an inhibitor, such as reported by Hayashi(10,11), does not play a substantial role in cessation of vascular inflammation, at least under the experimental conditions in our study. It should be noted that a fresh, acute reaction developed in vessels about which mononuclear cells, without doubt left over from the previous reaction, were abundant. This occurred even when small amounts (27 μg NBSA) of antigen were used. It is very possible that the inhibitor reported by Hayashi, which he indicates is related to the mononuclear cells, is overwhelmed by the inflammatory reaction that occurs following fresh antigen-antibody combination in vessel walls and with the subsequent influx of polymorphs.

Thrombosis of vessels in the Arthus reactions unquestionably plays an important role in development of the macroscopic lesion, and resolution of these thromboses is most certainly a factor in termination of the reaction as a whole. However, from previous studies in which the Arthus vascular inflammation was prevented by depletion of polymorphs(2), few cellular thromboses occurred indicating that thrombosis comes about secondary to the inflammatory reaction of the vessels. Moreover, that the thromboses resolve secondary to decreasing vascular inflammation seems most likely.

Summary. 1) Our studies suggest that neither exhaustion of humoral or cellular factors necessary for inflammation, nor presence of possible inhibitors of the reaction play a dominant role in diminution of inflammation and healing of a hypersensitivity vasculitis of the Arthus type. 2) This study supports the importance of ridding the diseased vessel of antigen and antibody in bringing about a decrease in reaction and healing.

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Lactogenic Hormone Requirements for Milk Secretion in Intact Lactating Rats.* (25066)

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Early studies indicated that lactogenic hormone released from the adenohypophysis in response to nursing stimuli plays an important role in maintenance of lactation(1,2,5). Recent experiments have indicated that lactogenic hormone is rather ineffective in maintaining milk secretion in hypophysectomized

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rats but when combined with other hormones about 50% of normal milk production was obtained (3,4). The possibility exists, as a result of these investigations, that lactogenic hormone is effective in maintaining lactation only when metabolic requirements for milk secretion have been met. It seemed of interest, therefore, to determine if a more accurate quantitative assessment of loctogen requirement for milk secretion could be made by using intact animals whose general metabolism was unimpaired.

Materials and methods. Adult primiparous lactating rats of Sprague Dawley-Rolfsmeyer strain were housed in individual cages in a room maintained at 78 ± 1°F and given free access to Purina Lab. Chow and water. Each litter was adjusted shortly after birth to 6 young. On day 14 postpartum the following procedure was employed to estimate amount of milk secreted by a lactating rat during 2 consecutive 12-hour periods: Each litter was isolated from their mother during each 12-hour period. At onset of first isolation, 3 additional young of comparable size and weight from donor mothers were added to each litter of 6. *Control.* Shortly after end of first isolation period each of 14 lactating rats was weighed to nearest .5 g and injected subc. with 1 unit oxytocin.‡ The young were immediately replaced and allowed to nurse. A second injection of 1 unit oxytocin was administered 10 minutes later. After the young had nursed a total of 30 minutes, each mother was reweighed. The same procedure then was repeated after a 2nd 12-hour period of isolation of mother and young. *Experimental.* Fifty-six lactating rats were injected i.p. with 4.5 mg/100 g Nembutal 20 minutes prior to end of first isolation period. A depth of anaesthesia was reached within 20 minutes whereby breathing became deep and regular and spinal reflexes were obtunded, *e.g.*, lack of flexor response when Achilles tendon or feet were pinched tightly. Each mother was weighed to nearest .5 g, laid on her side and her young replaced. One unit oxytocin was injected i.p. a few minutes after the young became attached to the teats. A second injection of 1 unit oxytocin was administered 10 minutes later. When it became apparent from behavior of the young that milk flow from mammary glands had ceased, the litter was removed and each mother reweighed. Five lactating rats were similarly treated except 2.5 mg/100 g Nembutal was administered, a level which resulted in loss of consciousness but without abolishment of spinal reflexes. Immediately following completion of nursing, mothers received a single subcutaneous injection of .2 ml saline or .5, 1, 1.5 or 2 mg lactogenic hormone§ each in .2 ml saline. In one group, however, a second injection of hormone was made 6 hours after the first. Following a 2nd 12-hour isolation of mother and young, each lactating rat was placed under light Nembutal anaesthesia and milked out as described for first nursing.

Difference in maternal body weight before and after each nursing was used to represent amount of milk secreted during previous 12 hours. All rats were examined at end of first nursing to ascertain whether all glands had been nursed. If one or more glands were not nursed the teats were covered with Scotch tape prior to 2nd nursing. In such cases amount of milk was calculated for 12 glands on basis of that obtained from nursed glands. All lactating rats were killed after 2nd nursing, skinned and their mammary glands examined for presence of milk. In a preliminary study 10 lactating rats were killed at completion of the first nursing and their mammary glands similarly examined.

Results. The technic of injecting 1 unit oxytocin 2 × at 10 minute intervals to conscious or anaesthetized lactating rats on day 14 postpartum, and using 9 young to nurse the glands, proved to be an effective means of evacuating almost all of milk which had been secreted during a previous 12 hour period. Gross examination of 10 rats after the first nursing in a preliminary experiment, and of all animals in the present study after the second nursing showed the mammary glands to be essentially devoid of milk. They were quite red in appearance with only an occasional splotch of white. Weight loss of moth-

‡ Kindly supplied by Armour Labs.: diluted to contain 1 unit/.2 ml.

§ Lactogenic hormone was a gift from Endocrinology Study Section, N.I.H.

TABLE I. Effect of Lactogenic Hormone upon Milk Secretion by Lactating Rats on Day 14 Postpartum. Lactogen administered after 1st nursing.

Treatment	No. of rats	1st nursing: Prenursing body wt (g)	1st nursing: Milk* (g)	1st nursing: Milk (g/100 g)	2nd nursing: Prenursing body wt (g)	2nd nursing: Milk* (g)	2nd nursing: Milk (g/100 g)	% of control
Control	14	306.9	18.9	6.16 ±.18	297.9	8.7	2.92 ±.19[1]	
Nembutal, 2.5 mg/100 g	5	323.4	20.4	6.30 ±.36	306.0	7.6	2.48 ±.22	
" , 4.5 "	11	301.0	18.9	6.27 ±.24	287.3	2.5	.85 ±.05[2]	29.1
Idem + .5 mg lactogen	10	315.7	19.5	6.18 ±.24	301.5	5.4	1.79 ±.14[3]	61.3
" + 1.0 mg lactogen	10	294.4	18.1	6.15 ±.25	283.1	5.7	2.01 ±.18[4]	68.8
" + 1.0 mg lactogen (.5 mg 2×)	7	302.1	18.1	6.00 ±.15	275.6	5.3	1.92 ±.18[5]	65.8
" + 1.5 mg lactogen	9	301.2	18.7	6.21 ±.22	284.1	5.0	1.76 ±.17[6]	60.3
" + 2.0 " "	9	321.3	20.8	6.47 ±.22	304.2	6.1	2.01 ±.17[7]	68.8
Mean	75	307.2	19.1	6.22 ±.05				

* Milk from 12 mammary glands removed by 9 nursing young with aid of oxytocin (1 unit 2× at 10 min. intervals).

Student's "t"	Probability
1–3,4,5,6,7	.025
2–1,3.4,5,6,7	.001
3–7	.5

ers proved preferable to weight gain by nursling young as an indication of amount of milk present in glands. Defecation or urination did not occur in mothers but invariably occurred in young once milk flow had commenced. Preliminary data also indicated 9 young were more effective than 10-12 for purposes of total milk withdrawal primarily because of less crowding and reduced competition for teats. With this technic an average of 19.1 g of milk was obtained from 75 lactating rats following initial 12 hours allowed for milk secretion. A significant positive correlation ($P = < .01$) existed between amount of milk obtained and maternal body weight. Values obtained thus were expressed as g/100 g body weight. On this basis, milk yield values were very uniform between groups and also between individuals in any one group (Table I).

Milk secreted during second 12-hour period by untreated lactating rats averaged 2.92 g/100 g or 47.2% of that obtained after the 1st 12-hour period (Table I). Administration of 2.5 mg/100 g Nembutal at time of first nursing had no effect upon amount of milk secreted during 2nd 12 hours. Rats injected with 4.5 mg/100 g Nembutal, a level which resulted in deep anaesthesia for about 1 hour, exhibited a significant reduction in average milk yield of .85 g/100 g following second 12-hour period. No overlap with control values occurred (Table II). Injection of .5, 1.0, 1.5, or 2.0 mg lactogenic hormone/rat at end of first nursing to deeply anaesthetized lactating rats evoked significant restoration ($P = .001$)

TABLE II. Distribution of 2nd Nursing Milk Yield Values from Table I.

Distribution of:	Avg milk (g/100 g): Nembutal range .7–1.1	Control range 1.4–2.3	Control range 2.4–3.3	Control range 3.4–4.3
Control		4	5	5
Nembutal, 4.5 mg/100 g	11			
Idem + .5 mg lactogen	2	7	1	
" + 1.0 mg "	1	6	3	
" + 1.0 mg " (.5 mg 2×)	2	2	3	
" + 1.5 mg "	1	7	1	
" + 2.0 mg "	2	4	3	
Total lactogen	8	26	11	

in milk secretion (Table I). Progressively greater milk yield did not result from administration of larger doses of lactogen or, in one instance, from dividing the dose and injecting 2 × at 6-hour intervals. Analysis of correlation coefficients indicated, however, that milk secretion resulting from injection of lactogen was significantly greater ($P = .01$) in those rats from which greater milk yields were obtained at first nursing. Average yield obtained from injecting any one level of lactogen was still significantly less ($P = .025$) than control value of 2.92 g/100 g. Administration of any one level of lactogenic hormone was not effective in all instances (Table II). One or 2 rats in each lactogen-treated group failed to secrete milk in excess of range of values obtained for saline-injected anaesthetized group.

Discussion. The role of lactogenic hormone in initiation of lactation is well known. However, the extent to which the hormone maintains or influences intensity of lactation is less clear. It has been suggested that regular application of milking or nursing stimuli are necessary for effective maintenance of lactation since such stimuli evoke rapid release of oxytocin from the neurohypophysis for milk removal(6) and of lactogen from the adenohypophysis for milk secretion(7). The observation that an appropriate depth of anesthesia effectively blocked pituitary discharge of both hormones in response to nursing stimuli in lactating rats(8) provides a technic whereby requirements of each hormone for milk secretion and removal in intact animals can be quantitatively estimated. Estimation of oxytocin requirements with this method has been reported(9). It has been substantiated in the present study that 4.5 mg/100 g Nembutal effectively prevents release of lactogen. Anaesthetized rats whose mammary glands have been emptied of milk with oxytocin failed to secrete appreciable quantities of milk during a subsequent 12 hour period but injection of lactogenic hormone to such rats restored milk secretion approximately 70% of normal. Attempts to maintain milk secretion in hypophysectomized rats with lactogen alone have not met with this degree of success. When combined with various other hormones only about 50% of normal milk production has been obtained(3,4). This suggests that replacement of hypophyseal hormones in the proper ratios has not yet been attained. It has been suggested that lactogen perhaps triggers enzyme systems involved in synthesis of milk from precursor substances(10,11). It would appear, therefore, that a proper balance of hormone influencing the well-being of the organism and regulating the supply of metabolites for milk synthesis must be present for lactogen to be fully effective. The results of the present investigation, in addition to confirming the concept that lactogen is vitally concerned in maintenance of lactation, lead to 2 additional considerations. The observation that lactogen was not equally effective in restoring milk secretion in lactating rats suggests that amount of lactogen reflexly released from the pituitary in response to nursing stimuli may limit the intensity of milk secretion in some animals. It follows that supplemental administration of hormone might improve lactation in such cases. The second consideration stems from inability of lactogen alone to produce 100% restoration of milk secretion. The possibility exists that Nembutal blocks release of other anterior pituitary hormones which normally might be reflexly released by stimulus of nursing and which might act synergistically with lactogen to effect full milk secretion. There is evidence that nursing stimuli during lactation induces release of ACTH(12,13) and it is quite possible growth and thyrotropic hormones are similarly affected.

Summary. A technic is described for determination of amount of lactogenic hormone required for milk secretion in intact lactating rats. Rats were isolated from their litters for 12 hours on day 14 postpartum then deeply anaesthetized with 4.5 mg/100 g Nembutal and all milk withdrawn by 9 young with aid of oxytocin. With lactogenic hormone discharge blocked and mammary glands empty, significantly less milk (.85 g/100 g maternal body weight) was obtained following a 2nd 12-hour isolation period in comparison with 2.92 g/100 g for unanaesthetized controls. Injection of .5, 1, 1.5 or 2 mg lactogenic hormone during 2nd isolation period significantly restored milk yield approximately 70%. Yields

were, nevertheless, significantly less than controls. No greater milk yield resulted from injection of more than 1 mg lactogen. These data indicate that lactogen is necessary for maintenance of milk secretion in intact rats and strongly imply that since lactogen alone was incapable of restoring milk secretion 100%, other pituitary hormones are necessary for full milk secretion and perhaps might be susceptible to discharge by nursing stimuli.

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Excretion, Enterohepatic Circulation, and Retention of Radiovitamin B_{12} in Pernicious Anemia and in Controls.* (25067)

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The routes and patterns of Vit. B_{12} excretion have been studied in animals(1,2) and in man(3,4,5). In human studies, some indications were found of an intestinal reabsorption of Vit. B_{12} excreted in bile. The purpose of the present paper is to confirm this finding and to extend the studies to patients with deficient B_{12}-absorption. Retention of B_{12} in serum, and B_{12} excretion in controls and in patients with B_{12}-deficiency were also studied.

Material and methods. Controls were 11 healthy, non-hospitalized males between 20 and 34 years old (normal controls), and 4 female and 2 male hospital patients between 16 and 69 years old (control patients). Their diagnoses were asthenia and achlorhydria, allergic bronchial asthma, diabetes mellitus, and acute or subacute nephritis (3 cases). Random serum samples showed normal B_{12} concentrations. All control patients had normal Vit. B_{12}-absorption tests (21-55%) by the technic described earlier(5). Four additional patients with a post-operative drain in the common bile duct were employed to obtain several bile samples within a short time. The 13 cases with disturbed B_{12} metabolism (Table I) had either an acute Vit. B_{12} deficiency as evidenced by low Vit. B_{12} levels in serum and by megaloblastic anemia reacting to B_{12} treatment, or disturbed Vit. B_{12}-absorption, mostly both. Case No. 4 is insufficiently studied and results based on material involving this case are in brackets (Table IV). Between 0.3 and 0.6 μg Vit. B_{12} labeled with Co^{56} or Co^{58} and with approximate specific activity of 2 mc/mg were injected intramuscularly. Two persons received 1 μg because of the decay of radioactivity. Twenty-nine serum samples were obtained from 17 persons between 3-17 days after injection of labeled B_{12}. All urine was collected from 24 persons during 3 days after injection. From 29 persons 136 24-hour feces collections were obtained, starting on the average 6.8 (4-10) days after radiovitamin injection. Feces were liquefied by nitric acid digestion and concen-

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TABLE I. Thirteen Cases with Disturbed B_{12} Metabolism.*

Sex	Age, yr	B_{12} absorption test Urinary excretion, % of given amt	Serum vit. B_{12}, $\mu\mu$g/ml	Megaloblastic bone marrow	Histamine fast gastric achlorhydria	Total body hemoglobin, g	Hb (g/100 cc)	RBC (mill.)	WBC (× 1000)
♂	49	2	165		+	560	12.6	4.0	5.6
♂	56	2	16	+	+	450	9.8	2.9	4.0
♀	69		40	+	+	433	12.8	3.7	5.3
♂	52				+	750	13.7	4.3	6.0
♂	26	11	115			405	9.1	3.8	7.0
♂	55	7	10	+	+	274	8.8	1.9	5.0
♀	45	9	165	+		365	8.2	2.3	3.8
♀	73	4	32		+	385	8.8	2.3	3.2
♂	67	6	14	+		350	8.6	2.4	6.1
♀	65		20	+	+	425	7.5	1.7	4.0
♂	62	5	170	+	+	448	4.7	2.5	3.5
♂	55		36	+	+	500	9.5	2.5	2.6
♂	59	1		+	+	230	5.6	1.3	4.5

* Space left empty = test not performed.

Clinical diagnoses were, in case 1 chronic gastro-duodenal-jejuno-ileitis with malabsorption syndrome, in cases 2-3, 6, 8-13, pernicious anemia, in case 4 chronic pancreatitis with malabsorption syndrome, in case 5 mesenteric and pulmonary tuberculosis with malabsorption syndrome, and in case 7 malabsorption syndrome.

trated by evaporation. Bile, unless otherwise stated, signifies strongly bile coloured mixture of duodenal juices obtained by duodenal lavage (kindly performed by Dr. G. Perman) (6). Fifty-nine bile samples and 11 samples of bilirubin-free duodenal juice were taken from 30 individuals 12.8 (7-19) days after isotope administration, and after collection of feces. In some cases a satisfactory flow was easily obtained, in others bile secretion had to be stimulated by cholecystokinin or magnesium sulfate. Bilirubin determinations were performed within a few hours after duodenal lavage. Bile samples were kept in the dark at +4°C and concentrated by evaporation. Methods of determining B_{12}-binding (Table III), bioassays, and scintillation measurements will be described elsewhere(7). Radioactivity measurement times were adjusted to keep statistical error of counts between 1 and 10%. Daily bilirubin production was estimated individually in each case, and employed to calculate daily B_{12} excretion in the bile. The percentage saturation of hemoglobin with CO (COHb) was calculated from determinations of CO pressure in alveolar air (9,10). Simultaneously, total amount of Hb (THb) was estimated by determining the increase in COHb after rebreathing of a known amount of CO in a closed system(10). From THb and COHb the Hb destruction rate (d.r.) was calculated(10). Daily bilirubin production could be roughly calculated to be = (THb x 1/120 x d.r. x 4 x 619 x 584/619) x 1.05/66.700 mg, where 1/120 is the fraction of Hb normally destroyed/day. The factor 1.05 is due to the part of bile pigment derived mainly from heme-enzymes(10). In some instances the d.r. could not be calculated since the persons had inhaled CO (for instance by smoking) before the experiment. In these cases, the d.r. was assumed to be 1 for controls and 2 for cases with B_{12}-deficiency. The figure 2 is the lowest value recorded in cases of untreated megaloblastic anemia which had not been exposed to CO inhalation, both in

TABLE II. Excretion of Vit. B_{12} in Controls and B_{12}-Deficient Patients.

Excretion	No. of cases	Controls	No. of cases	B_{12}-deficient
Urinary, total excretion 1st-4th day	14	12.8 ± 1.87%*	6	8.7 ± 1.93%*
Fecal, daily excretion, later than 4th day	16	.5 ± .17%†	9	.3 ± .06%†
Biliary, daily excretion, later than 4th day	14	1.5 ± .31%†	8	.4 ± .11%†

* % of inj. dose. † % of inj. dose *minus* loss 1st-4th day. Means ± S. E. of mean.

TABLE III. Vitamin B_{12}-Binding Capacity* in Serum.

Controls	B_{12}-deficient patients
2.9 ± 1.16 mμg/ml (4 cases)	3.2 ± .67 mμg/ml (8 cases)

Means ± stand. errors of mean.

* Nondialysable after adding 200 mμg radio B_{12} per ml serum.

present and in previous materials(10). Daily radiovit. B_{12} excretion was estimated to be = C x (radiovit. B_{12} concentration) x (calculated daily bilirubin production) / (bilirubin concentration in sample). Since only a part of daily radiovit. B_{12} excretion in bile is measured, the factors by which this part is multiplied to obtain total daily excretion must not be too large. An attempt was made to correct for the fraction of bile pigments not giving the diazo reaction, and for the probable maximum amount of duodenal radioactivity not originating in pure bile. This correction constant (C) was calculated to be 0.76(7).

Results. Retention of Vit. B_{12}. Mean daily excretions of radioactivity in both urine, bile and feces were numerically higher in controls than in cases with subnormal Vit. B_{12} levels (Table II) indicating that more of the injected B_{12} is retained by B_{12}-deficient patients. Similarly, these patients show higher serum concentrations of radioactivity than the controls (Fig. 1). No significant difference could be observed, however, in B_{12} binding capacity between the 2 groups of sera (Table III). Distribution of all excretion values was markedly skew, and the difference is statistically significant only as regards biliary excretion ($0.005 > p$), and serum concentration (Fig. 1).

Excretion routes. It has been demonstrated (3,4) that, physiologically, most Vit. B_{12} in man is excreted by the fecal route, and that Vit. B_{12} reaches the gut mainly with the bile. The present study shows that radioactivity is present not only in bile, but also in other constituents of duodenal juice. Mean radiovitamin concentration in bile (*i.e.*, duodenal juice mixed with bile) was 19 times higher than that in bilirubin-free duodenal juice.

Enterohepatic circulation. Radioactivity calculated to be excreted daily in bile was larger in control cases than the amount/day in feces (Table IV). In spite of large individual variations, the difference between mean daily biliary excretion and mean fecal excretion is statistically significant ($0.01 > p > 0.005$). Likewise, the mean of the differences is significantly ($0.005 > p$) higher than zero. In patients with deficient Vit. B_{12} absorption, on the other hand, daily excretion in bile was smaller than or equal to fecal excretion in about half of the cases (Table IV). The difference between mean biliary excretion and mean fecal excretion is not statistically significant in this group.

Discussion. Distribution of radioactivity in the body resembles that of B_{12}(12). There is evidence that radioactivity in liver derives from microbiologically active Vit. B_{12}(11). Such facts have been accepted previously(11) as indications that radioactivity really represents B_{12}. For this reason radioactivity was calculated as Vit. B_{12}. The standard deviation of B_{12}/bilirubin ratios in 39 samples obtained from 8 patients was 26% of corresponding mean values. Time lapse between 2 samples was 1 hour, 1 month. In comparing

TABLE IV. Enterohepatic Circulation of Vitamin B_{12} in Controls and in Cases with Deficient Vitamin B_{12} Absorption.

Group of subjects	Biliary excretion No. of cases	Biliary excretion %/day*	Fecal excretion No. of cases	Fecal excretion %/day*	No. of cases with indications of enterohepatic circulation
Normal controls	9	1.5 ± .36	10	.5 ± .23	9
Control patients	5	1.4 ± .59	6	.5 ± .16	5
Total controls	14	1.5 ± .31	16	.5 ± .17	14
Cases with deficient B_{12} absorption	11 (12)	.9 ± .31 (.9 ± .30)	12 (13)	.3 ± .05 .4 ± .07	6 (6)

* % of inj. dose *minus* loss during 1st-4th day.

Means ± stand. errors of mean.

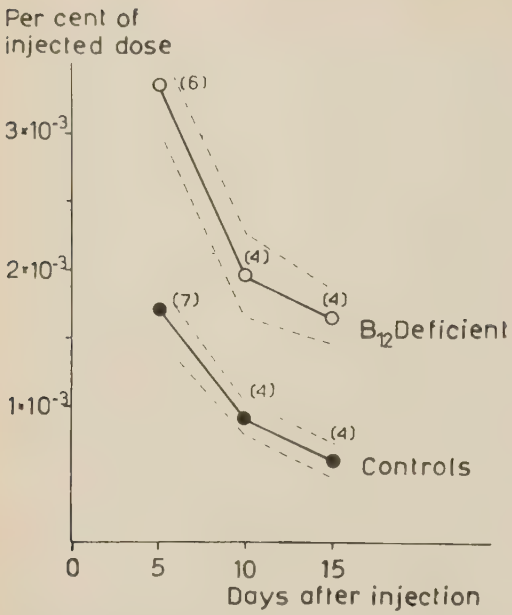

FIG. 1. Serum radioactivities after radiovitamin administration. Differences between controls and B_{12} deficient cases are statistically significant; $p < 0.005$ (15 days), $0.5 > p > 0.02$ (5 and 10 days). ——— means, – – – – – stand. error. Figures in parentheses indicate No. of samples.

controls with patients with B_{12}-deficiency, the difference in age and sex should be taken into account.

Vit. B_{12} retention. Previous studies on retention of Vit. B_{12} in patients with B_{12}-deficiency as compared to controls have yielded controversial results(11,13,14), possibly due to composition and size of control materials (14) or to therapeutic amounts of B_{12} given to pernicious anemia patients prior to experimental period(11). The present data suggest that, under present experimental conditions, more B_{12} may be retained by patients with Vit. B_{12}-deficiency. At any rate, more of the radio-B_{12} in the body is in the serum of these patients than in controls, indicating that in B_{12}-deficiency serum B_{12} might constitute a larger part of total body B_{12} than under normal conditions. Since the amount retained in serum is very small compared to B_{12}-binding capacity, even significant differences in the latter(13,15) are unlikely to explain differences in retention.

Excretion routes. Previous studies in rats with ligated common bile ducts(3) and in dogs with biliary fistulae(1) indicate that Vit. B_{12} can reach the gut by another route besides the biliary one. The results can be confirmed by present investigations in man under more physiological conditions. It appears probable that the major part of non-bilirubin-containing duodenal juice consists of pancreatic juice. This could be consistent with the observation of a high radioactivity in the pancreas of a pig receiving injections of radiovit. B_{12}(7).

Enterohepatic circulation. The fact that calculated biliary excretion of radioactivity is consistently and considerably higher than fecal excretion favours the concept of an enterohepatic circulation. If estimated daily bilirubin production, and thus daily B_{12} excretion, are too high this could simulate an enterohepatic circulation. However, the mean B_{12}/bilirubin ratio in controls was 35 $\mu\mu$g radio-B_{12}/mg bilirubin and mean daily fecal excretion in the same cases was 2000 $\mu\mu$g radio-B_{12}/day. If no enterohepatic circulation were present, this would correspond to a mean daily excretion of bilirubin of less than 60 mg/day, which is impossible. Finally, in one case of pernicious anemia in relapse (case No. 13) more radioactivity was actually collected by duodenal lavage within an hour than was excreted/day in the feces. Duodenal lavage was performed after completing feces collection. No attempts to express quantitatively mean daily excretion of B_{12} to the gut by other routes than the biliary are justified by present studies since it is difficult to estimate the daily volume of such excreta. However, existence of this extra-biliary excretion implies that the calculated amount of radioactivity reaching the upper part of the intestine is minimal.

Summary. 1) After parenteral administration of radiovit. B_{12}, radioactivity reaches the gut mainly with the bile, but other components of duodenal juice also contain radioactivity. 2) The calculated daily biliary excretion of radioactivity in controls is invariably larger than daily fecal excretion, suggesting enterohepatic circulation. In cases with defective B_{12}-absorption, there are indications of an enterohepatic circulation in only about half of the cases. 3) A higher percentage of radio-

vitamin administered is retained in serum, and a numerically lower percentage is excreted/day in urine, bile, and feces of patients with B_{12}-avitaminosis than in controls. These facts suggest that more B_{12} is retained in states of deficiency.

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Effect of Relaxin on Mammary Gland Growth in Female Mice.* (25068)

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In a previous paper it was reported that relaxin in synergism with estrogen stimulated lobule-alveolar growth of mammary gland of intact and castrate male mice pretreated with diethylstilbestrol to stimulate extensive duct growth(1). The extent of gland development induced experimentally was checked by whole mounts of the glands and by determination of total desoxyribosenucleic acid (DNA) content of mammary glands. The present report concerns the synergistic role of relaxin and estrogen in stimulating growth of the lobule-alveolar system of intact female, ovariectomized, and ovariectomized-hysterectomized mice. For comparison, the type of growth and DNA content of mammary gland of comparable mice injected with estrogen and progesterone are presented.

Procedure. Female virgin albino mice weighing approximately 28 g were fed a commercial mouse feed. The single or concomitant doses of relaxin, estradiol benzoate, and progesterone were injected subcutaneously on the back in 0.1 ml of olive oil daily for 10 days. On 11th day mammary glands were removed. Seven glands (4 posterior and 3 anterior) were analyzed for DNA by Webb and Levy method(2), and 3 anterior glands were prepared for whole mounts for morphological examination. Relaxin‡ was washed with anhydrous ether to remove possible progesterone contamination, dissolved in small amount of distilled water, lyophilized and suspended in olive oil by homogenization.

In one group the ovaries were removed whereas in a second group both ovaries and uteri were removed. Experiments were started 12 days after surgery.

Results. Intact female mice injected with estradiol benzoate 0.75 μg daily had mammary glands containing a mean of 2.541 mg

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‡ Relaxin preparations, W1164A-Lot 53, kindly supplied by Dr. R. L. Kroc, Chilcott Lab., Morris Plains, N. J. (assay 30 GPU/mg).

TABLE I. DNA Content of Mammary Gland of Female Mice Treated with Ovarian Hormones.

Group	Dose of hormones*	No. of mice	Dry, fat-free tissue wt (mg) Mean ± S.E.		DNA (μg)/dry, fat-free tissue (mg) Mean ± S.E.		Total DNA (mg) Mean ± S.E.	
Intact mice	E.B., .75 μg	19	63.6	2.2	40.2	1.2	2.541	.093
	Idem + R, .62 GPU	8	72.3	4.1	37.2	1.2	2.599	.202
	" + " 1.25 "	9	69.9	4.4	38.1	2.2	2.659	.246
	" + " 2.5 "	8	76.4	3.3	37.2	1.5	2.838	.150
	" + " 5.0 "	17	68.1	7.1	42.9	1.5	2.886†	.102
	R, 5.0 GPU	9	56.8	4.2	44.4	2.0	2.477	.161
	E.B., .75 μg + P, .75 mg	9	58.2	2.9	45.4	2.6	2.648	.202
	Idem + " 1.5 "	8	60.1	5.0	44.3	1.2	2.658	.213
Ovariectomized mice	E.B., .75 μg	9	61.8	4.9	37.6	1.1	2.343	.224
	Idem + R, 5.0 GPU	10	69.4	2.6	35.4	.7	2.449	.077
	" + P, .5 mg	10	68.6	2.7	35.6	1.1	2.430	.096
	" + " .75 "	10	66.4	1.0	37.9	3.1	2.510	.131
Ovariectomized-hysterectomized mice	E.B., .75 μg	10	63.0	2.8	38.5	.9	2.434	.124
	Idem + R, 5.0 GPU	10	60.1	4.7	42.8	2.4	2.605	.244
	" + P, .75 mg	9	66.9	3.2	39.3	1.5	2.645	.195
	" + " 1.0 "	10	65.0	3.0	40.5	1.1	2.620	.106

* E.B. = estradiol benzoate; R = relaxin; P = progesterone.
† Significant at 2% level from group inj. estradiol benzoate alone.

of DNA per 7 glands. This amount of estrogen concomitant with graded levels of relaxin stimulated mammary glands containing a mean maximum level of 2.886 mg of DNA which is significantly greater than the estrogen control. In comparison, mammary glands of mice on 2 levels of progesterone contained amounts of DNA comparable to the lower levels of relaxin (Table I). The group receiving relaxin alone were not different from the group on estrogen alone.

The whole mounts of glands of mice stimulated with estrogen in some cases showed slight lobule-alveolar growth. Mice stimulated with estrogen and graded doses of relaxin presented a progressive lobule-alveolar development up to the highest dosage. The type of gland development in these groups was essentially the same as that stimulated with estrogen and progesterone.

Mammary glands from ovariectomized and hysterectomized mice injected with estrogen contained less mean total DNA than intact mice. Further, the groups on estrogen and relaxin had less mean total DNA than corresponding intact groups. The groups on estrogen and varying levels of progesterone showed that 0.50 mg per day is less effective than 0.75 or 1.00 mg in promoting mammary gland growth.

In the above groups, removal of the ovaries caused involution of the glands. Injection of estrogen alone stimulated only duct growth, whereas the combinations of estrogen and relaxin or progesterone produced lobule-alveolar growth. The latter development was usually related to the total mean DNA.

Discussion. These data confirm and extend our previous observations(1) with male mice indicating that estrogen and relaxin in suitable amounts has the capacity of stimulating growth of the lobule-alveolar system of the mammary glands of intact, ovariectomized and ovariectomized-hysterectomized female mice comparable to that obtained by estrogen and progesterone as indicated by total mean DNA and histological examination of whole mounts of glands. Neither estrogen nor relaxin alone, in the amounts injected, stimulated lobule-alveolar growth.

In comparison with the male study, it will be noted the extent of total duct development was much greater in females based upon mean total DNA. Thus, in intact male mice total DNA after estrogen administration was 1.572 mg compared to 2.541 mg in intact females. Comparable data on males and females after estrogen and 5 GPU of relaxin are 2.019 and 2.886 mg respectively.

Primiparous mice at 6, 12, and 18 days of

pregnancy exhibit mean total DNA of mammary glands of 2.853, 4.118 and 5.969 mg. Thus, estrogen and relaxin stimulated lobule-alveolar growth as indicated by DNA equalled that of 6 days of pregnancy(3).

In the 2 groups ovariectomized, a period of involution of 12 days was allowed. Restimulation with estrogen did not restore the total DNA level to that shown by the intact animals. Neither was estrogen and relaxin able to stimulate total DNA equal to that of the intact groups. This difference is not considered as related to the lack of ovaries or uteri, *per se*, but rather to the effect of gland involution before hormones were begun. Since hysterectomized mice showed slightly greater total DNA than did the corresponding ovariectomized groups, the uterus has no inhibitory action on the effect of relaxin on mammary gland growth.

Summary. Intact, ovariectomized, and ovariectomized-hysterectomized mice were treated with estrogen, estrogen and relaxin, and relaxin alone for periods of 10 days. It was shown that estrogen and higher levels of relaxin synergize in the promotion of lobule-alveolar growth as indicated by total DNA of the glands and by histological examination of whole mounts. Estrogen or relaxin alone did not stimulate such growth. Mean total DNA at optimal levels of relaxin was equal to that observed in mice pregnant 6 days. In the operated groups, reduced total DNA may be due to gland involution of 12 days.

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Method for Determination of 17,21-Dihydroxy-20-Ketosteroids in Urine and Plasma.* (25069)

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The adrenal cortex secretes several steroids directly associated with human metabolism. Metabolism, separation, measurement and identification of these steroids have been the subject of much research. A quick, accurate method of measurement of these steroids is of great value to the clinician for correct diagnosis and treatment. Porter and Silber(1) reported that a solution of phenylhydrazine in sulfuric acid formed a colored product when mixed with 17,21-dihydroxy-20-ketosteroids. Using this color reaction Nelson and Samuels (2) formulated the first practical method for routine quantitative determination of 17-hydroxycorticosteroids in blood. By their procedure the blood extract was purified by solvent partition and chromatography on a synthetic magnesium silicate (Florisil) column. Several other methods for determination of free plasma 17-hydroxycorticosteroids have since been developed. Sweat(3) described a procedure involving chromatographic analysis of pure mixtures of several steroids on a silica gel micro column. Quantitative recovery of steroids was determined by technics employing both fluorescence and reaction with phenylhydrazine. A simple, accurate method for determination of the 17,21-dihydroxy-20-ketosteroids in plasma and urine has now been devised. It consists in a method of extraction similar to that of Paterson(4) and a procedure for chromatographic resolution on silica gel columns similar to the one reported by Sweat (3).

Materials. 1) A 1 ml serologic pipette is used as the column. 2) Glass wool (washed repeatedly with redistilled absolute ethanol)

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is placed into 1 ml serologic pipette and firmly packed until top of glass wool forms a tight, flat pad. 3) Silica gel (Davison, 100-200 mesh size) is washed with redistilled absolute methanol and supernatant is discarded. After washing 11 additional times, silica gel is dried beneath incandescent light (100 watts) overnight and kept in desiccator. 4) Chloroform is redistilled immediately before use. 5) Control samples of plasma came from a group of women and men aged 18 to 35 years. *Chemical assay.* 1) Color development with phenylhydrazine-sulfuric acid solution (6.5 mg phenylhydrazine hydrochloride/10 ml of 7.3 N sulfuric acid) is brought about by incubation in water bath at 38°C for 3 hours. 2) Samples are read in Beckman quartz spectrophotometer at 410 mu in 0.5 ml micro cuvettes. *Extraction.* Plasma (6-20 ml) or urine is adjusted to pH 1, with concentrated hydrochloric acid, extracted 3 times with 2 volumes of freshly distilled chloroform; the combined chloroform extracts are back extracted 2 times with 0.1 volume of 0.1 N NaOH followed by 2 washings of 0.1 volume of distilled water. The chloroform extracts are dried with anhydrous sodium sulfate, evaporated to dryness by distillation *in vacuo,* transferred quantitatively to small test tube and dried with stream of nitrogen. *Chromatography.* 100 mg of silica gel is added to 5 ml of chloroform, placed in serologic pipette with a capillary tube, packed by gentle tapping and washed with additional 8 ml of chloroform. The dried extract of plasma is dissolved in 1 ml of chloroform and placed on silica gel with a capillary tube. The test tube is washed with 8-1 ml portions of chloroform, these washings placed on column and resulting effluent is collected as fraction one. The column is further developed with 3 ml of 0.2% solution of absolute ethanol in chloroform and the effluent is collected as fraction 2. The third and last eluant consists of 5 ml of a 10% solution of absolute ethanol in chloroform. The last 2 fractions are divided into 2 equal aliquots (using aliquot of each fraction as a blank), dried and assayed by procedure previously described.

TABLE I. Mean Percentage of Recovery with Varying Amounts of Hydrocortisone Added to Plasma (5 Exp.).

No. of samples	Added hydrocortisone (μg)	% recovery (mean)	% recovery (range)
3	.60	80	78-82
3	1.17	83	80-86
6	3.53	85	83-88
3	3.30	89	87-92
4	4.25	92	90-95

Vol of plasma used in extraction, 10 ml.

TABLE II. Recoveries of Hydrocortisone from Plasma in 11 Tubes.

Exp.	Added hydrocortisone (μg)	P-S* chromogens (μg)	Amt recovered (μg)	% recovery
1	6.62	7.69	5.99	90
2	"	7.77	6.07	92
3	"	7.44	5.74	87
4	8.50	9.68	7.98	94
5	"	9.36	7.66	90
6	0	1.58		
7	0	1.78		
8	0	1.73		
9	0	1.65		
10	0	1.70		
11	0	1.60		

Vol of plasma used in extraction, 20 ml.

Exp. 1-5: reproducibility.
6-8: non-partitioned extract.
9-11: partitioned " .

* 17,21-dihydroxy-20-ketosteroids.

Results. Chromatography of hydrocortisone, cortisone and Reichstein's compound S resulted in recoveries of 90 to 96%. When the compounds were added to plasma, extracted and chromatographed, recoveries ranged from 80 to 94%.

Table I shows mean percentage of recovery in control experiments in which different amounts of hydrocortisone were added to pooled plasma. Recoveries were higher with larger amounts of hydrocortisone. Lines 1 and 2 illustrate recovery that may be expected from normal plasma by this method, since our values in 19 normal subjects ranged from 6 to 13 μg/100 ml of plasma.

Table II contains recovery data of hydrocortisone and results of representative experiments in which determinations of 17,21-dihydroxy-20-ketosteroid group of pooled plasma were made before and after partition of chloroform extracts between a 70% solution of aqueous ethanol and low boiling petroleum ether. The values for Exp. 1 through 5 illustrate the

TABLE III. Urinary and Blood Values of 3 Patients with Cushing's Disease and Adrenal Hyperplasia.

Urinary K-S	Urinary formald.	Urinary P-S chromogens	Plasma P-S chromogens	Plasma P-S chromogens after ACTH
mg/24 hr			μg/100 ml	
23.70	1.68	3.07	30	
12.80	2.34	2.88	43	67
39.50	11.95	53.60	81	96

reproducibility of this procedure. Exp. 6 through 8 represent initial amounts of 17,21-dihydroxy-20-ketosteroids in plasma. Exp. 9 through 11 represent samples that were partitioned between low boiling petroleum ether and a 70% solution of aqueous ethanol. All chloroform extracts gave positive reactions to Tollen and blue tetrazolium tests. The reactive material migrated at the same rate as hydrocortisone on paper chromatograms(5).

Table III contains results obtained in 3 patients with diagnoses of Cushing's disease and adrenal hyperplasia subsequently confirmed at operation. Column 4 gives initial high values of plasma Porter-Silber chromogens with increase after administration of ACTH (column 5).

When urinary extracts obtained from 20 ml of normal urine were chromatographed according to method described, the values were approximately 13 to 20% less than those of corresponding unchromatographed samples. This is probably due to removal of impurities which tend to give a false high value for the dihydroxy-acetone group since chromatographic recovery experiments show a loss of only 4 to 7%. Recoveries of tetrahydrocortisone added to urine ranged from 87 to 102%.

Fig. 1 illustrates relative values obtained when formaldehydogenic and Porter-Silber determinations are performed on the same urinary extract. The values are below 1 mg/24 hr in most instances.

Discussion. Partial purification of plasma and urinary extracts is sometimes a desirable preliminary step for determination of 17-hydroxy-corticosteroids, although it can be time-consuming. A quick, accurate procedure for their determination is highly desirable. By applying our procedure to plasma and urine samples, 6 to 8 determinations are completed in a day without difficulty. Using twice the volume of chloroform shortens the time needed for extraction and results in few instances of emulsion formation.

Thirty μg of steroid could be chromatographed on silica gel columns described. When mixtures of cortisone, hydrocortisone and Reichstein's compound S (10 μg each) were chromatographed, there was only partial resolution of the compounds.

In routine analysis of 24 hour urine collections for formaldehydogenic compounds and 17,21-dihydroxy-20-ketosteroids determinations are carried out on the same chloroform extract. When urinary extracts are chromatographed and 17,21-dihydroxy-20-ketosteroids are determined quantitatively by Porter-Silber procedure, results are invariably lower than formaldehydogenic results. This is explained by the fact that only corticosteroids having 17,21-dihydroxy-20-keto side chain will give a positive reaction to the Porter-Silber test, whereas all types of 21-hydroxy corticoids will give a positive formaldehyde test.

Summary. A simple, accurate method for determination of the 17,21-dihydroxy-20-ketosteroids in plasma and urine has been described. The chloroform extract is dissolved in chloroform and chromatographed on silica gel in a 1 ml serologic pipette. The 17,21-

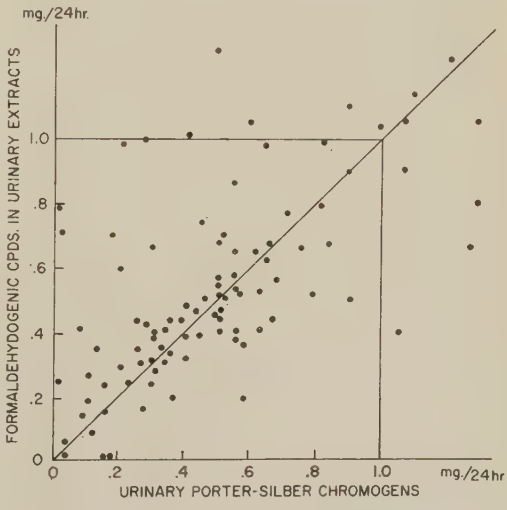

FIG. 1. Urinary Porter-Silber chromogens *vs* formaldehydogenic compounds in urinary extracts.

dihydroxy-20-ketosteroids are eluted with ethanol-chloroform mixtures and assayed by a micro Porter-Silber procedure.

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Inosine Effects on Plasma Inorganic Phosphate Movements in Fresh and Cold-Stored Blood.* (25070)

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Our purpose was to determine changes in phosphate movement in fresh and cold-stored blood in the presence of inosine, a substance currently used in clinical preservation of whole blood by cold storage. Influx of phosphate from plasma to the red cell has been studied several times in both fresh and cold-stored blood(1,2,3) but the efflux of phosphate from the cellular phase to plasma has been studied only in fresh blood(4,5,6). Inosine and other purine ribosides are metabolized by human erythrocytes(7,8,9) and this is associated with an increase in intracellular phosphorylated compounds. These and other intracellular metabolic changes doubtless influence the movements of phosphate between plasma and cells. We studied movements into and out of red cells by following changes in 1) plasma inorganic phosphate and 2) P^{32} in plasma and cells. To limit the number of variables, we added only anticoagulant and the reagents under study.

Methods. Blood from adult male donors was handled as described by Kahn and Acheson(10) except that glucose was omitted. At least 3 experiments were performed for each treatment. I. One hundred ml of whole blood either fresh or cold-stored was divided in half, and Sodium Radiophosphate (Abbott Labs) was added to one-half of the blood. Both samples of blood were incubated in constant temperature oven at 37°C for 75 minutes with intermittent gentle agitation. The 2 samples were then centrifuged at 0-4°C and the plasmas removed and saved. The cells were washed once with cold saline to remove most of trapped plasma. After centrifugation of the resuspended cells, the saline was discarded and the cells with P^{32} were added to the plasma without P^{32}, while the cells without P^{32} were added to the plasma with P^{32}. After the plasmas were exchanged, blood was added to glass-stoppered Erlenmeyer flasks containing 0.1 volume of either saline (control), or 40 mM inosine. The flasks were then shaken 3 hours at 37°C in a Dubnoff shaker. Samples were taken at different intervals and measurements of plasma radioactivity and inorganic phosphate were made. Zero time was the time the reconstituted blood was added to the flasks. II. In other experiments fresh blood was preincubated with P^{32} in the manner just described, but both samples (radioactive and non-radioactive) were stored in the cold room for 6 days before centrifugation, exchange of plasmas and 37°C incubation. A Beckman Model G pH meter measured pH of whole blood. Both fresh and cold-stored blood, containing inosine or saline, measured before, during and after the experiment, had pH's between 7.6 and 7.7. Hematocrits were measured in duplicate by the technic of Guest and Siler(11). When standard errors of the mean or probabilities were determined, the "t" test

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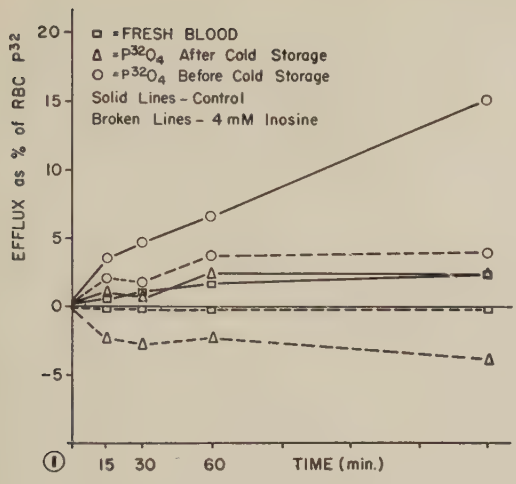

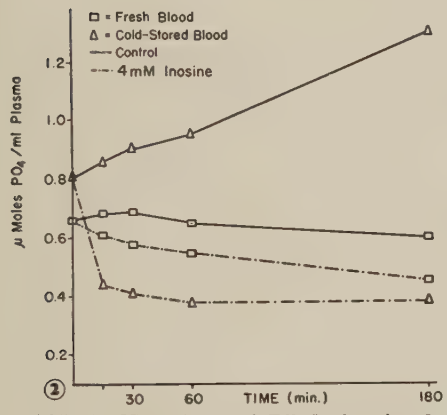

FIG. 1. Net efflux of P^{32} during incubation at 37°C.

FIG. 2. Plasma inorganic phosphate during incubation at 37°C.

was used(12). The method of counting P^{32} was that described by Kahn(3) and the method of determining inorganic phosphate was the Martin and Doty(13) modification of method of Berenblum and Chain(14).

Results. A: When fresh or cold-stored cells are loaded with P^{32} and resuspended in plasma initially free of P^{32} according to method I, an intracellular P^{32} concentration at least 10 times higher than that of the plasma is obtained. With further incubation at 37°C there is a measurable loss of P^{32} from cells to plasma. Both in fresh blood and in cold-stored blood this loss occurs within the first hour of incubation and is unchanged after 2 further hours incubation (Fig. 1). The loss amounted to less than 5% of radioactivity of cells (at hematocrits between 25 and 35). This result is similar to that previously found(4,5). In contrast, red blood cells which had P^{32} present during the 6-day cold-storage period (method II) always lost more than 15% of red cell P^{32} to the plasma (Fig. 1). Inosine, 4 mM, decreased the net loss in all experiments; when method I was used, this change was enough to cause a net gain of P^{32} by cells from the plasma (Fig. 1).

B: The movement of P^{32} from plasma into cells was consistent with findings already reported in fresh blood(1,2,3). In fresh blood with no inosine present 64 ± 1.2% of plasma P^{32} left the plasma during 3 hours of incubation and in cold-stored blood 32.6 ± 1.1% moved from plasma to cells. Inosine in fresh blood had no effect on loss of P^{32} from plasma in fresh blood, but in cold-stored blood it decreased this loss. The latter is in agreement with Kahn's result(3).

C: To evaluate the role of hemolysis, hemolysate was added to blood and distribution of P^{32} (added as orthophosphate) between the inorganic phase (butanol-benzene fraction) and the organic residue in plasma, was measured. After 3 hours of incubation, hemolysis did not affect this distribution in control flasks or with added glucose (20 mM). In the presence of inosine, however, hemolysis caused a rapid incorporation of P^{32} into organic phosphate in the plasma. Using the method of Kaplan and Greenberg(15) we found this to be primarily glycerophosphate. When there was more than the equivalent of .01 ml of packed red cells hemolyzed/ml of plasma, 80% of P^{32} of plasma was incorporated as plasma organic phosphate in less than 15 minutes at room temperature.

D: In fresh blood there is 10% fall in plasma inorganic phosphate during 3 hour incubation, whereas cold-stored blood had a 70% rise in plasma inorganic phosphate. Added inosine, strikingly decreased amount of plasma inorganic phosphate in cold-stored blood, where there was noticeable hemolysis. In fresh blood the decrease was smaller (Fig. 2).

E: In 13 experiments with fresh and cold-

TABLE I. Effects of Inosine on Intracellular Inorganic Phosphate (μMoles/ml Packed RBC) $\pm$ S.E.M.

Treatment	Fresh blood		Cold-stored blood	
	Before incubation	After 3 hr incubation	Before incubation	After 3 hr incubation
None	1.01 $\pm$ .09	1.86 $\pm$ .23	.40 $\pm$ .037	2.02 $\pm$ .229
1 mM inosine		.96 $\pm$ .337		1.11 $\pm$ .10
4 mM "		.46 $\pm$ .10		.44 $\pm$ .09

stored bloods, treated in the same way as previous experiments, except they were not subjected to preliminary incubation, incubation at 37° for 3 hours increased intracellular inorganic phosphate. Inosine at 4 mM final concentration decreased intracellular inorganic phosphate below the initial value in both fresh and cold-stored bloods (Table I).

Discussion. Inosine decreased net loss of P^{32} from plasma of cold-stored blood in Kahn's work(3) and in ours. In both cases cold-stored blood probably contained close to 1% hemolysis. This amount of hemolysis together with inosine can cause a measurable formation of plasma organic phosphate (section C). It appears that inosine decreases the net loss of plasma P^{32} by forming organic phosphate compounds in plasma containing this P^{32}, *i.e.*, by diminishing the inorganic P^{32} available for movement into the cells.

The marked drop in plasma inorganic phosphate in cold-stored bloods when inosine is added (Fig. 2) is probably due to formation of plasma organic phosphate. This fall was not as marked in fresh blood where there was little hemolysis.

To study the contribution of cellular phosphate to changes in plasma inorganic phosphate, we used cells containing high concentrations of P^{32}. Using cells loaded with P^{32} immediately before the test incubation, we found no difference between fresh and cold-stored blood. The most striking result was the larger gain of P^{32} by plasma when P^{32} had been present for 6 days during cold-storage (Fig. 1). We propose that there are at least 2 sources of P^{32} (or phosphate) to move out of the cell. One would be a small compartment, less than 5% of red cell P^{32}, which is rapidly turning over; this we propose is cellular inorganic phosphate. The larger compartment would be the cellular organic pool of phosphates. Brief exposure of cells to P^{32} would label the smaller compartment and most of this P^{32} would enter the cellular organic pool. After this loading period, the relatively small loss to plasma of recently acquired P^{32} suggests that the anabolic processes place the phosphate in compartments from which little of it escapes to the inorganic phase. P^{32} present for 6 days, even at cold-room temperature, would be distributed more evenly through the organic compartments, including the principal compartments subject to catabolism. Accordingly, the increase of P^{32} outflow when Method II is used would represent more uniform labeling of phosphate emerging from the organic pool.

Since, in presence of hemolysis, inosine promotes formation of plasma organic phosphate, we would expect inosine added to cold-stored blood to cause a greater net loss of P^{32} from cells because of the P^{32} retention by plasma compounds. Just the opposite is seen, *i.e.*, inosine decreases the net appearance of P^{32} from the cells into the plasma. This is true in fresh as well as cold-stored blood. Since inosine reduces flow of P^{32} into the cells in cold-stored blood, the observed effect of inosine on plasma P^{32} must underestimate retention of phosphate by the cells. Retention of cellular phosphate is consistent with the idea that purine ribosides are excellent substrates for human red cells(8). Cold-stored cells would show a greater effect with inosine because they lack substrate and therefore use more of the inosine. In the small cellular compartment, which we believe to be inorganic phosphate rapidly exchanging with plasma, almost all the phosphate would form ribose phosphate. Subsequent formation of other phosphorylated intermediates would divert the phosphate from leaving the cell. This expectation is confirmed by results of section

E where it is shown that inosine (which has previously been demonstrated to increase cellular organic phosphate(1)) sharply decreases cellular inorganic phosphate.

Summary. Movements of $P^{32}O_4$ in and out of fresh and cold-stored human erythrocytes were measured in presence and absence of inosine. I. Small amounts of hemolysis when inosine was present caused a decrease in plasma inorganic phosphate and incorporation of P^{32} into plasma organic phosphate. This formation of plasma organic phosphate decreased movement of P^{32} into the cell. II. When P^{32} was originally present in the red cell, inosine decreased the net movement from cells to plasma. We attribute this effect to utilization of inosine as substrate in the cell. III. Cells containing P^{32} during 6-day cold-storage lost 3 to 4 times as much P^{32} as cells having P^{32} for a short time. Movements from cells to plasma are described as the result of at least 2 phosphate compartments within the cell.

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Rate of Acetic Acid Production in the Rumen Determined by Isotope Dilution.* (25071)

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The fact that fermentation of carbohydrates in the rumen produces large quantities of short-chain fatty acids which are important sources of energy for the ruminant animal(1) has led to *in vitro*(2), isolated rumen(3), perfused rumen(4), and *in vivo*(1,5) technics of estimating the rates of production of these acids. To obtain better estimates of acetate production in the practically-fed ruminant, an isotope dilution method has been used in the work reported here.

Methods. A mature sheep fitted with a rumen cannula was maintained on an all-clover hay ration with minerals and water free-choice. *Via* a rumen cannula, 2.267 mc of sodium acetate-1-C^{14} was given 20 minutes after withdrawal of the morning hay. Samples of rumen liquor were then withdrawn through the cannula from the ventral-sac of the rumen near the ventral curvature, at approximately half-hour intervals over a 12-hour period. Immediately following withdrawal, each sample was strained through cheesecloth, the pH was lowered to approximately 1.5 with 10% H_2SO_4, and the sample was quick-frozen in dry ice and stored at 0°F until analyzed. Acetic, propionic, butyric, and the higher fatty acids were separated quantitatively by

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TABLE I. Distribution of Radioactivity/Milliliter of Rumen Liquor in Each Acid Fraction after Administration of Acetate-1-C^{14}.

Time after C^{14} acetate admin., min.	Thousands of dpm/ml of rumen liquor				
	Higher acids	Butyric acid	Propionic acid	Acetic acid	Total
15	.28	6.3	1.1	18	192
33	1.09	19.0	2.2	266	288
77	1.47	20.8	3.7	207	233
125	1.37	19.6	4.3	137	163
195	1.55	19.0	4.8	97	123
247	1.09	15.1	5.2	74	95
335	1.01	10.9	4.9	44	61
455	.65	5.9	3.3	25	35
720	.24	1.3	2.6	9	13

chromatography on Celite(6), and radioactivity of each acid fraction was determined in a liquid scintillation counter.

Results. Radioactivity was found in all 4 fatty acid fractions showing that considerable interconversion of acetic acid to butyric, propionic and higher fatty acids takes place in the rumen. Distribution of radiocarbon among the various rumen volatile fatty acids at time intervals following administration was determined and representative data are shown in Tables I and II. Total radioactivities and specific activities of the various fractions were in the order, acetic>butyric>propionic> higher acids. On the basis of the relative radioactivities of each acid when at their peaks (approximately ½ hour for acetic acid, 1 hour for butyric acid, and 3 hours for propionic acid) the percentage of acetic acid being converted to butyric would appear to be approximately 12% and to propionic acid, approximately 5%. Dilution experiments, similar to these with acetic acid, will have to be carried out with the other acids before more accurate inter-conversion data can be calculated.

TABLE II. Specific Activities (dpm/mMole) of Different Rumen Volatile Fatty Acids at Intervals following Rumen Administration of Acetate-1-C^{14}.

Time after C^{14} acetate admin., min.	Thousands of dpm/mMole			
	Higher acids	Butyric acid	Propionic acid	Acetic acid
15	95	935	57	2,390
33	369	226	108	3,369
77			260	2,633
155	480	2,204	259	2,033
221	669	1,905	280	1,314
247	500	3,246	358	1,153
335	382	1,716	265	645
455	440	1,177	266	543
720	125	335	238	206

Concentrations of the various fatty acids, expressed as millimoles of acid per liter of rumen liquor, are given in Table III. The rates of change of total radioacetic acid and of specific activity of radioacetic acid yielded straight line logarithmic functions while total acetic acid concentration yielded a straight line arithmetic function. The following regression lines were calculated for these 3 rates: (a) for disintegrations per minute of acetate per ml of rumen liquor, $\log y = 5.46007 - 0.002385\ x$; (b) for specific activity

TABLE III. mMoles/Liter of Rumen Liquor of Different Rumen Volatile Fatty Acids at Intervals after Acetate-1-C^{14} Administration.

Time after C^{14} acetate admin., min.	mMoles				
	Higher acids	Butyric acid	Propionic acid	Acetic acid	Total mMoles/l
15	2.95	6.71	19.6	77.1	106.4
33	2.64	8.41	20.7	78.9	110.6
77			14.4	78.7	
155	2.85	8.88	16.7	67.6	96.0
221	2.31	9.95	17.2	74.2	103.7
247	2.18	4.66	14.5	64.2	85.5
335	2.65	6.38	18.4	68.1	95.5
455	1.48	5.04	12.4	46.8	65.7
720	1.89	3.90	10.9	45.1	61.8

of acetate, $\log y = 6.57433 - 0.002123 x$; (c) for total acetate concentration per ml of rumen liquor, $y = 0.08152 - 0.00006292 x$; where x is time in minutes following administration of radioacetate.

The rate of change of specific activity of acetate, that is, rate of dilution, was used to compute the rate of acetic acid production. The decrease in specific activity of the radioacetic acid, due to dilution by acetic acid synthesized calculated over the straight line part of the curve (33 minutes to 455 minutes), was found to be at the rate of 50% per 130 minutes. On the basis of the total acetic acid present per ml of rumen liquor at the start of this period (33 minutes after radioacetic acid administration) and this rate of dilution over the 7-hour straight line period of isotope dilution, it was calculated that 0.126 millimole of acetic acid were synthesized per ml of rumen liquor per 7 hours. This amounts to 0.00756 g or 0.0264 Kcals of acetic acid. If one assumes a rumen content of 5 liters, this then would amount to 132 Kcals, which is approximately 30% of a 140 lb. sheep's 7-hour maintenance requirement.

Summary. It has been found by the technic of isotope dilution using acetate-1-C^{14} that the normal sheep on an all-hay ration produced acetic acid in its rumen by bacterial synthesis at the rate of 0.126 millimole per ml of rumen liquor for the 7-hour period following feeding. Radioactivity was found in the other rumen volatile fatty acid (propionic, butyric and higher acids) demonstrating their formation from acetic acid in the rumen.

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Effect of Methylphenidate on Cardiovascular Actions of Pressor Amines. (25072)

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Fröhlich and Loewi showed that cocaine potentiated pressor responses to epinephrine (1). Later, Tainter and Chang found cocaine blocked tyramine pressor responses(2). More recently, Fleckenstein and Bass(3) and Fleckenstein and Stöckle(4) demonstrated that cocaine altered responses of cat nictitating membrane to a series of sympathomimetic amines of the phenylalkylamine type, in such a way that contractions produced by one group of amines were augmented, contractions elicited by a second group were not affected and contractions produced by a third group were decreased. Recently Maxwell, *et al.*(5) demonstrated that the central nervous system stimulant, methylphenidate, potentiates pressor actions induced by epinephrine and norepinephrine while blocking and antagonizing pressor responses to amphetamine and ephedrine, respectively. In consideration of this, and the fact that the structures and primary pharmacological characteristics of cocaine and methylphenidate are markedly different, an attempt has been made to ascertain whether the effects of methylphenidate on cardiovascular actions of various phenylalkylamines would closely parallel those of cocaine.

Materials and methods. Mongrel dogs of both sexes weighing 7 to 15 kg were used. The anesthesia was Na pentobarbital, an initial dose of 30 mg/kg intraperitoneally fol-

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TABLE I. Effect of 15 mg/kg Methylphenidate I.V. on Mean Blood Pressure of the Dog.*

Control B.P. (mm Hg)	B. P. after methylphenidate (mm Hg)
120	115
130	120
100	100
120	100
110	100
90	100
105	105
100	100
120	120
115	115
111 ± 3.86†	108 ± 2.81†

* The difference between avg values was significant only at the 43% level by "t" test. The authors consider a level of significance of 5% or better to be required.
† Avg ± S.E.

lowed by 0.1 mg/kg/min infusion throughout the experiment. Blood pressures were determined by an Anderson glass-capsule manometer(6). Injections were administered intravenously. All drugs were in aqueous solution. Experiments were conducted in two ways: 1) An initial low dose of each pressor amine selected to produce a blood pressure elevation of at least 50 mm Hg was given. Fifteen to 30 minutes later 15 mg/kg of methylphenidate HCl was administered. Seven to 10 minutes elapsed before repeating the initial doses. In cases where pressure responses to repeated control doses of amines were blocked, dosage was increased 2 to 40 times to induce the return of amine responses to control levels. When repeated control responses were potentiated or were not altered by methylphenidate, no multiple doses were given. 2) To rule out the possibility of tachyphylaxis in studies of amines other than catecholamines, experiments were run in which the effects of initial doses and doses of amines given only after methylphenidate administration, were obtained in separate animals.

Results. Effect of methylphenidate on blood pressure. Administration of methylphenidate caused no significant change in mean blood pressure. Table I is a comparison of mean blood pressure levels before and after 15 mg/kg methylphenidate I.V. in 10 anesthetized dogs.

Pressor amine responses potentiated or not affected by methylphenidate. Pressor response to d - 1 norepinephrine HCl 4γ/kg, 1-epinephrine bitartrate 4γ/kg, epinine HCl 25γ/kg, cobefrine HCl 4γ/kg, synephrine tartrate 0.5 mg/kg and neosynephrine HCl 25γ/kg were obtained in 3 to 4 animals for each amine. All responses were at least 50 mm Hg. After administration of 15 mg/kg of methylphenidate, the above doses of amines were repeated with results that pressor responses to norepinephrine (Fig. 1A), epinine and cobefrine were clearly potentiated, while pressor actions of epinephrine, synephrine and neosynephrine were not significantly affected (Fig. 1A and Table II). These 6 amines will be referred to as Group I.

Pressor amine responses reversibly blocked by methylphenidate. Control pressor responses to ephedrine HCl 0.6 mg/kg, propadrine HCl 0.5 mg/kg and paredrine HBr 0.25 mg/kg were obtained in 4 to 9 animals for each amine (Table II). The doses were repeated in 2 to 7 of these animals after injection of 15 mg/kg methylphenidate and their effects were markedly antagonized. This blockade, however, could be overcome by increasing the doses of amines from 2 to 20 times (Fig. 1B). It is interesting that the blockade of ephedrine by methylphenidate could be overcome by increasing the dose level, despite the fact that animals are well known to exhibit tachyphylaxis to ephedrine.

In the second type of experiment 2 to 3 dogs were given above doses of ephedrine, propadrine and paredrine, for the first time, only after administration of methylphenidate. As in above experiments the pressor responses were markedly diminished, and this antagonism could be overcome by increasing the doses of amines from 2 to 20 times. Since the results of both experiments were similar the total number of animals following pretreatment with methylphenidate were grouped together and analyzed in Table II.

These 3 amines will be referred to as Group II.

Pressor amine responses irreversibly blocked by methylphenidate. Control responses to amphetamine sulfate 0.3 mg/kg, methamphetamine HCl 0.5 mg/kg, vonedrine HCl 0.5 mg/kg, tyramine HCl 0.25 mg/kg

TABLE II. Effect of Methylphenidate on Pressor Amine Responses.

Amine	Dose/kg (γ)	Controls: No. of animals	Controls: Mean pressor response ± S.E. (mm Hg)	Methylphenidate pretreated, 15 mg/kg I.V.: No. of animals	Methylphenidate pretreated, 15 mg/kg I.V.: Mean pressor response ± S.E. (mm Hg)	Level of signif. of differences in pressor responses before and after methylphenidate*† (%)
Group 1			x_1		x_2	
Norepinephrine	4	5	85 ± 12.04	5	135 ± 13.04	2
Epinephrine	4	7	80 ± 11.95	7	106 ± 9.91	13
Epinine	25	7	73 ± 8.79	7	109 ± 10.49	2
Cobefrine	4	5	75 ± 9.75	5	113 ± 7.00	1.4
Synephrine	500	4	86 ± 21.64	4	79 ± 20.65	80
Neosynephrine	25	3	67 ± 12.02	3	73 ± 4.42	60
Group 2						
Ephedrine	600	6	85 ± 10.88	4	11 ± 3.75	< .1
Propadrine	500	7	93 ± 5.86	5	20 ± 2.42	"
Paredrine	250	9	85 ± 8.29	9	2 ± 1.21	"
Group 3						
Amphetamine	300	4	91 ± 13.90	4	1 ± 1.25	< .1
Methamphetamine	500	5	73 ± 2.00	4	1 ± 1.25	"
Vonedrine	500	6	77 ± 13.27	4	1 ± .72	.7
Tyramine	250	6	94 ± 13.74	4	4 ± 2.40	< .1
Phenylethylamine	500	4	108 ± 12.50	5	10 ± 1.58	"
Paredrinol	250	6	98 ± 15.79	4	1 ± 1.25	.1

* Changes considered significant when level of significance is equal to or better than 5%.

† Significance of results determined by "t" test of Snedecor(9) $t = \frac{x_1 - x_2}{S_{x_1 - x_2}}$.

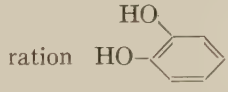
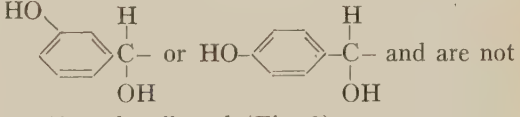
x = mean.

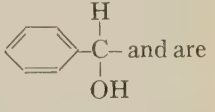
(Fig. 1C), phenylethylamine 0.5 mg/kg and paredrinol 0.25 mg/kg were obtained in 4 to 7 animals for each amine. In 2 to 3 of these animals, for each amine doses were repeated after injection of 15 mg/kg of methylphenidate and the responses were severely inhibited. However in contrast to amines in Group II this blockade could not be overcome by increasing the dose levels from 2 to 40 times (Fig. 1C).

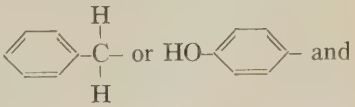
In a second set of experiments, comprising 2 animals for each amine the above doses were given for the first time only after methylphenidate administration. The results were similar to those where the amines elicited markedly reduced pressor responses which could not be surmounted by increasing the dose levels. Since the results of both experiments were similar, all animals following pretreatment with methylphenidate were grouped together and analyzed as a unit (Table II).

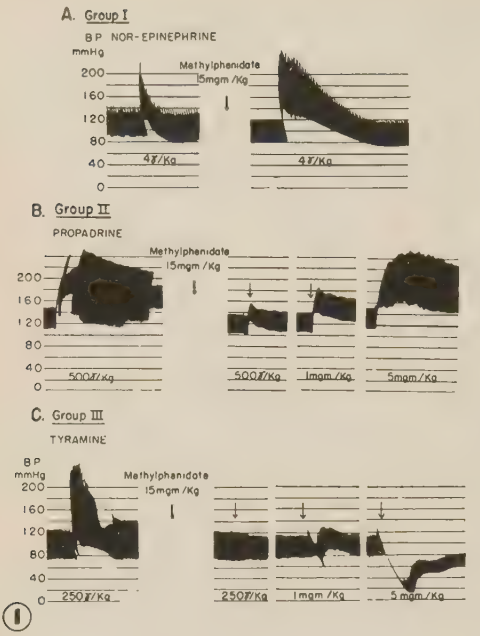
These amines will be referred to as Group III.

Discussion. Our experiments show that methylphenidate alters cardiovascular responses to a series of pressor phenylalkylamines in 3 ways: 1) by potentiating or not affecting amine pressor responses; 2) by reversibly blocking amine pressor responses; 3) by irreversibly blocking amine pressor responses.

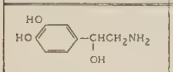
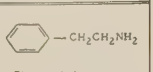
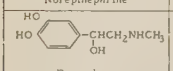
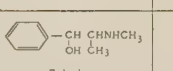
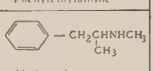
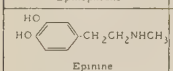
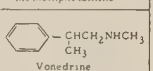
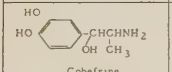
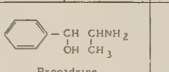
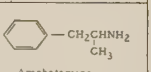
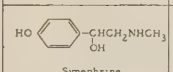
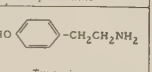
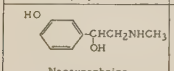
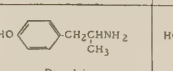
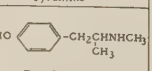
The amines in group I possess the configuration  and are augmented, or  and are not significantly affected (Fig. 2).

Group II amines possess  and are blocked but this antagonism can be overcome by giving higher doses (Fig. 2).

Group III is composed of amines with the configurations 



(1)

Potentiated or Not Affected (Group I)	Reversibly Blocked (Group II)	Irreversibly Blocked (Group III)
 Norepinephrine		 Phenylethylamine
 Epinephrine	 Ephedrine	 Methamphetamine
 Epinine		 Vonedrine
 Cobefrine	 Propadrine	 Amphetamine
 Synephrine		 Tyramine
 Neosynephrine	 Paredrine	 Paredrinol

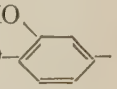
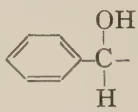
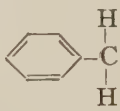
(2)

FIG. 1. Effect of intrav. methylphenidate on pressor responses to phenylalkylamines in mongrel dogs, anesthetized with pentobarbital.

FIG. 2. Effect of methylphenidate on pressor responses to sympathomimetic amines.

are blocked to an extent which cannot be overcome by administration of higher doses (Fig. 2). Paredrine with its configuration containing HO–  is the only sympathomimetic amine which has a structure belonging in group III but is affected by methylphenidate as are Group II amines (Fig. 2). No obvious explanation can be given for this seemingly anomalous occurrence.

This differential effect of methylphenidate, a central nervous stimulant, on pressor amine responses assumes more generalized importance with the knowledge that 3 other compounds of different primary pharmacological action and chemical configuration, also influence the activity of pressor amines similarly:

1) Fleckenstein and Bass(3) and Fleckenstein and Stöckle(4) have shown that intravenous injections of cocaine, a local anesthetic, affects responses of cat nictitating membrane to amines in such a way as to create 3 classifications. They are: 1) pyrocatechol derivatives possessing  which are enhanced, 2) intermediate substances with  which are not essentially affected and 3) neurosympathicomimetics containing  which are greatly antagonized.

2) Recently 2 compounds have been shown to affect pressor amine responses in almost identical manner as methylphenidate. One is the anti-hypertensive agent hexahydro-1-azepinepropionamidoxime (Su-4029) demonstrated by Maxwell *et al.*(7). The other reported by same authors(8) is the tranquilizing agent, reserpine.

The degree of similarity in influence of these 4 drugs on pressor amine activity is very notable. The structures of these compounds and their prominent pharmacological characteristics, as yet, offer no clue as to this parallelism.

Summary. Methylphenidate alters the pressor responses in the dog to a series of phenylalkylamines. The amines affected by this central nervous system stimulant are influenced in such a way as to fall into 3 distinct categories, *i.e.*, 1) potentiated or not al-

tered, 2) reversibly blocked and 3) irreversibly blocked. The differences in structure of pressor phenylalkylamines comprising the 3 groups are reviewed along with reference to other compounds which affect pressor action of these amines in a similar manner.

Grateful acknowledgement is expressed to Dr. Robert A. Maxwell for his valuable advice.

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Effect of Oxygen Tension on Sodium Transport Across Isolated Frog Skin. (25073)

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The transport of the sodium ion across the frog skin is an example of active transport (Huf, Wills and Cooley(1); Ussing and Zerahn(2)) and requires an adequate supply of oxygen. Production of the necessary energy depends on the integrity of oxidative enzymes in the skin. Cass(3) has shown that high oxygen tensions (8-35 atmos.) inhibit carbon dioxide production by frog muscle, and Gerschman *et al.*(4) have postulated that high oxygen tensions, like ionizing radiation, damage enzyme systems, possibly by increasing the concentration of free radicles in the tissue. The present study was undertaken to see whether a high pressure of oxygen inhibits sodium transport in the frog skin as it does other vital processes.

Method. The preparation used was essentially that described by Huf *et al.*(1). The skin was stripped from the hind legs of a frog and tied off at the distal ends to make 2 bags. Each bag was suspended "inside out" from the end of a piece of glass tubing inserted in the neck of the bag. Well-aerated Ringer's solution was introduced into the skin bag and the bag was completely immersed in similar Ringer's solution. The levels of the 2 solutions were the same inside and out to avoid hydrostatic effects. The preparation from one leg of each frog was placed in a simulated bomb kept at room pressure and temperature (19 $\pm$ 1°C) for 24 hours as a control and the skin bag from the other leg was placed for an equal period in the same room in a bomb where the pressure was elevated to 8-12 atmospheres using either 100% oxygen or 2.5% oxygen in nitrogen. Gas was introduced slowly over a 20 minute period to minimize rise in temperature due to compression. All gas that entered the bomb was passed through a carbon dioxide absorber to avoid depression of sodium transport by high carbon dioxide tension(2). The volume and sodium content of the Ringer's solution in the bag was measured at beginning and end of experiment. The area of the skin was measured by placing it on squared paper and counting the squares. Sodium analysis was carried out by flame photometry. The transport of sodium out of the bag was calculated as microequivalents per square centimeter per hour. (μ eq/cm^2/hr.).

Results. The transport in each experimental preparation was calculated as a per-

TABLE I. Statistical Analysis and Summary of Results.

Condition	Control	x	s	n	x vs 100%		x vs N_2 8-10 atm.	
					t	p	t	p
1. O_2 8 atm.	.143 ± .086	65.4	10.6	11	10.9	.001	13.7	<.001
2. O_2 8, 10, 12 "	.153 ± .076	57.4	10.9	15	15.1	.001	10.9	"
3. N_2 8, 10 "	.134 ± .050	111.5	17.8	7	1.7	0.15		

The means, x, are in % of 1 atm. air control taken as 100%.

centage of that in its paired control. The means (x) of these values (Table I) and their standard deviations (s) were found when (1) oxygen pressure equals 8 atmospheres, (2) oxygen pressure equals 8, 10, and 12 atmospheres, and (3) nitrogen pressure equals 8 or 10 atmospheres. The means (x) were first compared to 100% (the air control); then the high oxygen experimental means were compared to the nitrogen experimental mean in terms of t-value (t) and probability (p). Number of experiments in each series is denoted by (n). In all cases the formula(5)

$$t = \frac{x_1 - x_2}{Sp\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

was used where Sp is the standard deviation of the pool.

There was a great variation in absolute value of the transport of sodium in different experiments. The average control with standard deviation is expressed in Table I in microequivalents of sodium per centimeter square per hour (μ eq/cm^2/hr.).

In conditions 1 and 2, mean transport in the high oxygen tension is significantly lower than the control values (1 atm. air) but in condition 3, the high nitrogen pressure without deprivation of oxygen had no significant effect. Furthermore, comparison of the effects of high oxygen tension and of high nitrogen tensions showed that mean transport was significantly lower in the former case.

Therefore, we may conclude that oxygen tensions in excess of 8 atmospheres markedly inhibit sodium transport in the frog skin and that high pressures of nitrogen with normal oxygen tensions (*i.e.*, high pressures *per se*) do not have this inhibitory effect. These observations are consistent with the idea that excess of O_2 interferes with the normal activity of oxidative enzymes and inhibits sodium transport by diminishing the supply of energy available for the purpose.

Summary. Oxygen at 8 to 12 atmospheres depresses the transport of sodium ion across the isolated frog skin to 57.4%. Addition of 8 to 10 atmospheres of nitrogen without oxygen deprivation had no such effect.

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Rapid Production and Detection of Insulin-Binding Antibodies in Rabbits and Guinea Pigs. (25074)

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Insulin has generally been regarded as weakly antigenic in man and animals(1,2,3). Yet it has been demonstrated that virtually all humans receiving insulin therapy for more than a few weeks develop circulating insulin-binding antibodies demonstrable by technics employing I^{131}-labeled insulin and paper elec-

* Fellow of Nat. Fn.

trophoresis(4). Although Arquilla and Stavitsky were able to immunize rabbits with alum-precipitated crystalline insulin(5), Lowell and Franklin(6) and Moloney and Coval(7) found it difficult to immunize rabbits with unmodified insulin in Freund adjuvants, whereas guinea pigs responded uniformly with antibody production(7). The present study reports uniform development of insulin-binding antibodies in guinea pigs and rabbits immunized with unmodified crystalline beef or crystalline pork insulin in a modified Freund adjuvant.

Methods. Crystalline beef or pork insulin§ in physiological saline (1 mg/ml) was homogenized at high speed in a Virtis Homogenizer for 1 minute with equal volume of adjuvant (⅔ mineral oil-Bayol F.†; ⅓ Aquaphor‡). Five rabbits (3-5 kg) were injected subcutaneously with .5 mg insulin 3-5 times/week for 1½ to 9 weeks. Seventeen guinea pigs (300-710 g) received one subcutaneous injection 0.1-0.25 mg insulin and occasionally a second injection 3-6 weeks later. Despite apparently complete emulsification of the insulin-adjuvant homogenate, 3 guinea pigs had fatal hypoglycemic reactions. Crystalline insulins were labeled with I^{131} by methods described(4) yielding specific activity of 3-24 mc I^{131}/mg insulin and average less than 1 iodine atom/molecule 12,000 M.W. insulin. Disappearance of radioactivity following subcutaneous injection of insulin-I^{131} with and without adjuvant was measured by gamma ray scintillation counter. Times for absorption of 50% of insulin-I^{131} were 1¾, and 2 hours without adjuvant and 4 and 12 hours with adjuvant for 2 experiments in each group (Fig. 1). The extent of binding of insulin by antibody was determined by paper electrophoresis or hydrodynamic flow paper chromatography in veronal buffer pH 8.6, employing insulin-I^{131}(4). When plasma or serum containing insulin-I^{131} is placed on Whatman 3 MM paper, unbound insulin is adsorbed to the paper

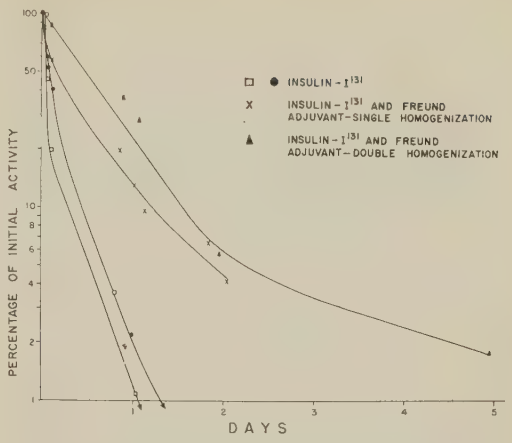

FIG. 1. Disappearance from subcut. inj. sites of crystalline beef insulin-I^{131} with and without modified Freund adjuvant.

at site of application ("origin"), whereas insulin bound to antibody migrates away from the origin with the binding protein(4). Homogenization with adjuvant produced no alterations in insulin-I^{131} detectable by paper electrophoresis, employing methods described elsewhere for detection of damage to I^{131} labeled proteins(4,8). All sera were incubated with the antigen for 4 hours at 37°C to allow maximum binding by the antibody.

Results. Insulin antibodies were demonstrable in all 5 rabbits within 11-36 days, and in all 14 surviving guinea pigs within 10-63 days after initial injection. On electrophoresis, insulin-antibody complexes in rabbit plasma migrated with the γ globulins (Fig. 2) and, in guinea pig plasma, in region of fibrinogen. Insulin-antibody complexes in human antiserum migrate in the inter β-γ zone(4). Normal guinea pigs, normal rabbits and rabbits immunized with human serum albumin or with rheumatoid arthritis factor showed no plasma binding of labeled insulin.

When insulin concentration is varied but antiserum concentration kept constant, the percentage of bound insulin decreases progressively with increase in insulin concentration, whereas the absolute quantity of bound insulin increases to an asymptotic value(4) which represents maximum binding capacity (Fig. 3). Maximum insulin-binding capacities ranged from less than 10 to 5000 mμg insulin/ml antisera in guinea pigs and from

§ We are indebted to Dr. O. K. Berens and Dr. C. W. Pettinga of Eli Lilly Co., Indianapolis, for generous gifts of crystalline beef and pork insulin.

† Bayol F. Imperial Oil Co.

‡ Aquaphor Duke Lab., Norwalk, Conn.

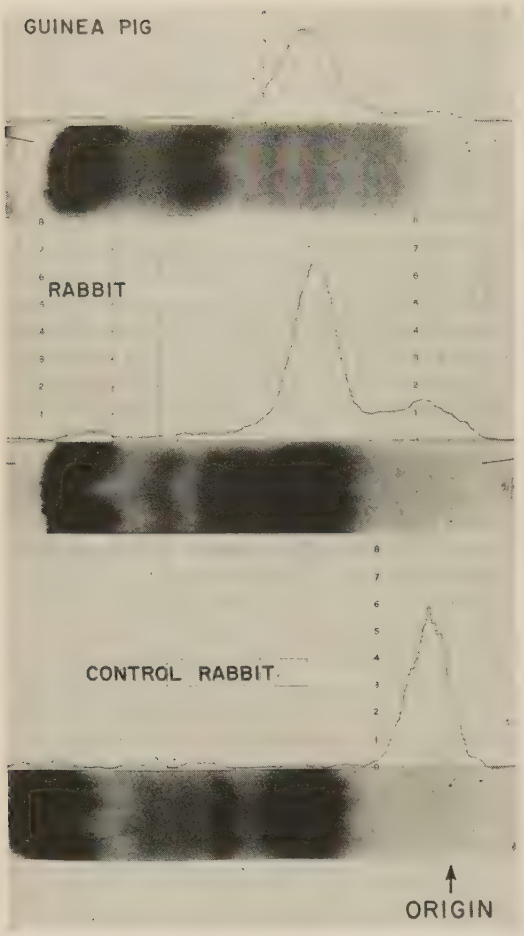

FIG. 2. Paper radioelectrophoretograms comparing migration of beef insulin-I^{131} in plasma of immunized guinea pigs (top) and rabbits (middle) and control rabbits (bottom).

14-550 mμg/ml in rabbits. Even much lower concentrations of antibody are detectable by these technics.

No precipitation was observed after 1 week of refrigeration in mixtures in which concentrations of bound insulin were 1/10th the maximum binding capacity of the antiserum.

Discussion. Successful demonstration of insulin-binding antibodies following immunizing procedure described here depends, first, upon use of a well emulsified, semi-solid preparation which markedly retards the otherwise rapid absorption of the small antigen from injection site, and secondly upon the very high sensitivity for detection of binding by antibody. Previous assays of antibody activity have included: resistance to insulin induced hypoglycemia in immunized rabbits(6), passive transfer of resistance in mice(7), systemic anaphylaxis in the sensitized guinea pig(9,10), complement fixation in rabbits(11), and agglutination of rabbit antisera with insulin conjugated rabbit and sheep erythrocytes(5). Sensitivity of these methods varies considerably and it is possible that the lowest titers of antibody noted here might well have gone undetected by other methods. Failure to demonstrate precipitating antigen-antibody complexes is in agreement with other authors(5) although concentrations of antigen-antibody complexes were frequently sufficiently low that, on this account alone, precipitation might not have occurred.

The effect of insulin-binding antibodies on response to hormonal action of insulin is currently under study. However, it must be emphasized that in most immunized animals total insulin-binding capacities were so low that significant insulin resistance is not to be anticipated.

Summary. Rabbits and guinea pigs were immunized with untreated crystalline beef and pork insulin in modified Freund adjuvant. Disappearance of insulin-I^{131} from subcutaneous sites of injection was markedly reduced by the adjuvant. Fourteen of 17 guinea pigs and all 5 rabbits survived otherwise lethal doses of insulin. Insulin-binding antibodies were produced within 11-36 days in all rabbits and within 10-63 days in all surviving guinea pigs. Insulin-antibody complexes in rabbit plasma migrate with γ globulins and in guinea pig plasma with fibrinogen,

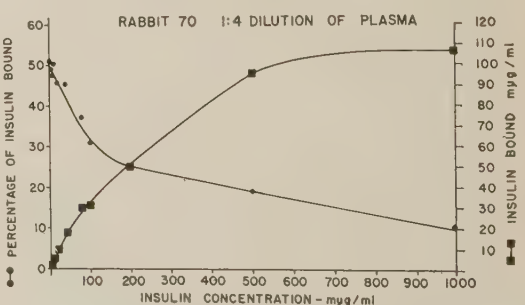

FIG. 3. Quantity of beef insulin-I^{131} bound (■) and percentage of beef insulin-I^{131} bound (●) in rabbit anti-beef insulin serum as a function of unlabeled beef insulin concentration.

on paper electrophoresis. Total insulin-binding capacities of antisera ranged from less than 10 to 5000 mμg/ml in guinea pigs and from 14 to 550 mμg/ml serum in rabbits.

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Hyperlipemia and Hemolysis III. Acceleration of Oleate Lysis of Human Erythrocytes by Homologous Plasma. (25075)

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In a previous study(1), human erythrocytes were found to compete *in vitro* with homologous plasma for binding exogenous sodium oleate. That portion of oleate bound by plasma was rendered hemolytically inert; even vigorous mechanical agitation failed to dissociate the binding appreciably. That portion of exogenous oleate bound by the erythrocyte constituted the active hemolytic moiety. The present report concerns the nature and mechanism of human plasma acceleration of hemolysis following erythrocyte-oleate binding; these findings extend earlier reports(2) indicating that once human erythrocytes bind oleate, homologous plasma reverses its hemolytic protective action and accelerates oleate lysis. Such studies are a continuation of investigations regarding possible intermediate pathways for *in vivo* hemolysis observed during conditions associated with rapid increases in plasma unesterified fatty acid(3-6). From our present findings, a scheme is inferred which may clarify certain events linking *in vivo* plasma unesterified fatty acid increases with subsequent hemolysis.

Materials and methods. Heparinized blood of all major groups was collected from normal human subjects, spun at 2500 rpm, 0°C, 15 minutes, and erythrocytes washed 3 times in sodium phosphate buffer, pH 7.2, ionic strength 0.15. Unless otherwise specified, standardized erythrocyte preparations were employed wherein addition of 0.5 ml of erythrocyte suspension to 2.5 ml of fatty acid or control solutions resulted in final concentration of 5 x 10^5 cells/cu mm. All fatty acid solutions were prepared in sodium phosphate buffer. The effect of temperature on pH of such buffered sodium oleate (Baker) solutions, measured with glass electrode, was as follows: 37°C, pH = 7.10; 25°C, pH = 7.20; 0°C, pH = 7.30. All fatty acid solutions were allowed to remain at room temperature at least 24 hours prior to use, since the hemolytic potency declined upon standing, rapidly at first, then more slowly. Human plasma albumin and globulin fractions were prepared from 3 individual citrated normal plasma specimens by overnight dialysis against ammonium sulfate solutions at 4°C employing magnetic stirring. Globulin-rich fractions were obtained at 50% ammonium sulfate saturation; albumin-rich fractions at 100% saturation. The fractions were washed with appropriate ammonium sulfate solutions, resuspended in buffered isotonic saline (pH 7.4) and cleared of ammonium ion (verified with Nessler's reagent) by dialysis at 4°C

TABLE I. Acceleration of Oleate Hemolysis by Plasma and Plasma Proteins.

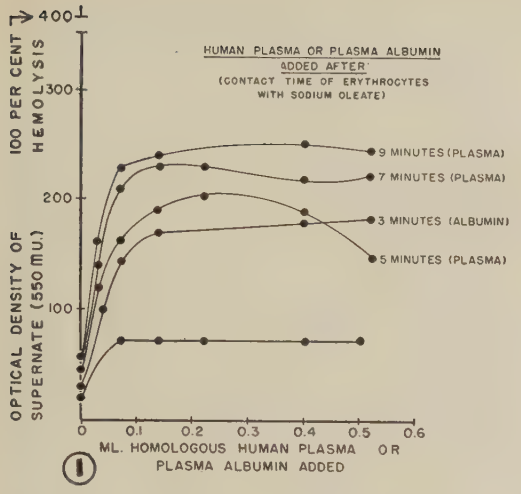
2.5 ml sodium oleate + 0.5 ml standardized human erythrocyte suspension +	Final oleate conc. ($\times 10^{-4}$ M) 4			2			1			0.5	
	°C										
	37	25	0	37	25	0	37	25	0	37	25
Isotonic buffered saline	1.1* (1.1)	1.5 (1.5)	18	1.5	3.0	50	2.0 (2.0)	5.0 (5.0)	350	9.0	22
Bovine serum albumin (40 mg/ml)	.16 (60)	.16 (60)	.50	.33	.42	.75	.75 (60)	1.5 (60)	90	6.0	12
Homologous human plasma	.25 (60)	.25 (60)	.75	.42	.50	1.5	1.0 (60)	1.5 (60)	90	7.0	12
Homologous human plasma albumin	.25 (4.0)	.25 (24)		.42	.50		1.0 (60)	1.5 (60)		7.0	12
Homologous human plasma globulin	.70 (1.7)	1.0 (3.2)		.83	1.0		1.0 (10)	3.5 (30)		8.5	16

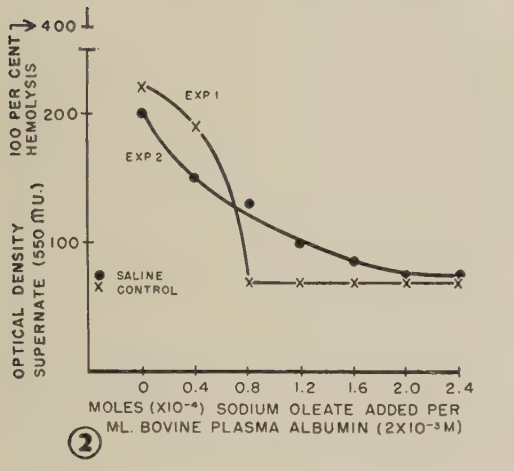
* Minimum oleate-erythrocyte contact time (min.) after which 0.10 ml of saline, plasma, or plasma proteins induce immediate 100% lysis. Figures in parentheses give 100% lysis time when sequence of combining is reversed, *i.e.*, when oleate and 0.10 ml saline, plasma, or plasma proteins are mixed first, followed by addition of erythrocytes. All values are means of 3 determinations.

against isotonic saline. Final volume of plasma fractions was adjusted to that of original plasma volume by addition of buffered isotonic saline. Cholesterol (Pfanstiehl) suspensions in the buffered saline were prepared by method of Lee and Tsai(8). Total cholesterol determinations in plasma and plasma fractions were performed according to method of Bloor(9).

Results. Acceleration of oleate hemolysis by plasma and plasma fractions. When a fixed number of washed human erythrocytes are suspended in sodium oleate, after a definite latent period, dependent at pH 7.2 ± 0.1 upon temperature and oleate concentration, addition of homologous plasma or plasma fractions induces immediate 100% hemolysis. Table I indicates *minimal latent period* required for immediate 100% lysis of standardized human erythrocyte suspensions by 0.10 ml homologous plasma and plasma fractions at varying oleate concentrations and temperatures. For example, 15 seconds *after* erythrocyte insertion in 2.5 ml sodium oleate (4 x 10^{-4} M, 37°C), addition of 0.10 ml homologous plasma induced immediate 100% lysis; such plasma addition to sodium oleate *prior* to erythrocyte insertion would have increased 100% lysis time from 1.10 to 60 minutes. From Table I it is apparent that minimal latent period required for immediate 100% lysis of standardized human erythrocyte suspensions by a fixed quantity of homologous plasma or plasma fraction is dependent upon initial oleate concentration and upon temperature; in general, the higher the oleate concentration or the lower the temperature, the relatively shorter the minimal latent period in relation to control 100% hemolysis time. Anticoagulants were not responsible for plasma hemolysis acceleration since homologous serum produced comparable results.

Intermediate phases of oleate lysis acceleration by human plasma. The preceding experiments indicate that a definite latent period is required following erythrocyte suspension in oleate solutions before addition of homologous plasma induces 100% hemolysis; the effect of plasma addition during the latent period was now determined. Small quantities (<0.10 ml) of homologous plasma or plasma albumin added rapidly to washed human erythrocytes suspended in 0.5 x 10^{-4} M sodium oleate at 25°C increased hemolysis in almost linear fashion; with larger plasma quantities, hemolysis attained a plateau or declined (Fig. 1). Time of oleate-erythrocyte contact prior to plasma addition determined the initial slope of the hemolysis curve as well as subsequent lysis plateau level. Under the same conditions, bovine serum albumin (2 x 10^{-3} M) and homologous human globulin produced similar families of hemolysis curves. Analysis of Fig. 1 suggests that as oleate-erythrocyte contact time increases, progressively more erythrocytes become "susceptible" to lysis by ho-





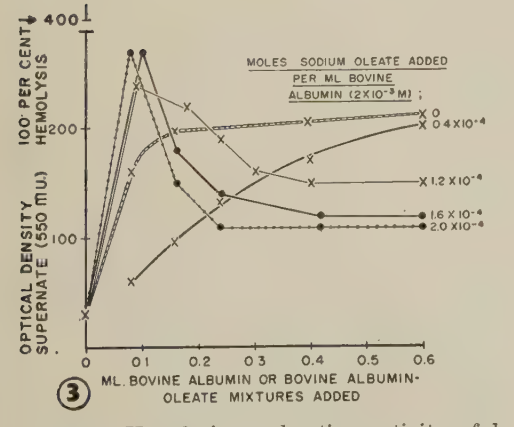


FIG. 1. Hemolysis accelerating activity of human plasma and human plasma albumin on homologous erythrocytes suspended in 3.0 ml sodium oleate (0.5×10^{-4} M, pH 7.2, 25°C). Lowest curve represents effect of plasma albumin addition 2 min. after erythrocyte-oleate contact. Each value is the mean of 4 determinations. (Optical density of supernates corrected for volume changes induced by varying quantities of human plasma or plasma albumin.)

FIG. 2. Effect of pretreatment of bovine serum albumin with sodium oleate on oleate hemolysis accelerating activity. Each test performed by adding 0.40 ml bovine serum albumin or bovine serum albumin containing added oleate to human erythrocytes suspended for 5 min. in 3 ml sodium oleate (1×10^{-4} M, pH 7.2, 25°C). Each value is mean of 4 determinations.

FIG. 3. Effect of concentration of bovine serum albumin-oleate mixtures on hemolysis accelerating activity for human erythrocytes suspended 5 min. in 3 ml sodium oleate (1×10^{-4} M, pH 7.2, 25°C). Each value is mean of 4 determinations. (Optical density of supernates corrected for volume changes induced by varying quantities of bovine serum albumin.)

mologous plasma and that this effect cannot be attributed simply to the increased control oleate lysis that occurs in the absence of plasma addition. Moreover, the initial linear relation between plasma quantity added and hemolysis (which can be demonstrated more precisely by studying the action of bovine albumin (2×10^{-3} M) on human erythrocytes in 1×10^{-4} M sodium oleate at 37°C) suggests a stoichiometric interaction between plasma factors and "susceptible" erythrocytes.

Nature of oleate hemolysis acceleration by homologous plasma. Two general mechanisms seem possible for oleate hemolysis acceleration by homologous plasma: 1) acceleration of *intrinsic* oleate lytic activity, or 2) acceleration of lysis by *superimposed* lytic mechanisms independent of intrinsic oleate hemolysis. Washed human erythrocytes were therefore exposed to increasingly dilute sodium oleate solutions at room temperature until thresholds were attained at which erythrocytes, after 5 saline washings, exhibited no increased tendency to lysis upon incubation at 37°C. Lysis of such erythrocytes by bovine or human albumin was not observed; erythrocyte binding of lytic quantities of oleate appears to be a prerequisite. Plasma and plasma fractions thus seem to act primarily by accelerating intrinsic oleate lytic activity, but this does not necessarily implicate the same intermediate pathways. Since oleate reduces aqueous surface tension, human plasma might accelerate erythrocyte lysis in oleate solutions by abruptly raising solution surface tension. This

possibility can be excluded since isotonic saline will raise oleate solution surface tension abruptly (measured with glass capillary tubes) without accelerating hemolysis; moreover, human erythrocytes pretreated with sodium oleate and washed repeatedly could be lysed immediately upon contact with homologous plasma as described previously(2). These observations do not exclude the possibility that abrupt surface tension alterations *within* the erythrocyte membrane may be concerned in the oleate hemolysis acceleration.

Inhibition of oleate hemolysis accelerating activity of human plasma. Pretreatment of human plasma or bovine serum albumin with increasing quantities of sodium oleate at 25°C progressively inhibited and finally abolished oleate hemolysis accelerating properties (Fig. 2). Such pretreatment simultaneously reduced the capacity to protect human erythrocytes from oleate hemolysis. By saturating the oleate binding sites, plasma and plasma fractions simultaneously lose ability to protect against or to accelerate oleate hemolysis. Incidentally, such findings strongly suggest that the oleate molecule on the surface of the erythrocyte membrane is directly responsible for lysis accelerating activity of homologous plasma. As anticipated from preceding findings, human plasma albumin, capable of binding more fatty acid than plasma globulin(10), exhibited greater oleate hemolysis protective and accelerating activity than the corresponding globulin fraction, (Table I). Nevertheless, oleate hemolysis protective and accelerating properties may not run parallel; thus whole human plasma afforded greater oleate hemolysis protection than did the corresponding plasma albumin fraction, yet was no more effective in accelerating oleate hemolysis, Table I. Although pretreatment of human plasma or bovine albumin with sodium oleate inhibits the oleate hemolysis accelerating mechanism, when such solutions are diluted with isotonic saline, the inhibitory effect wanes. Indeed, a sufficiently dilute solution of bovine albumin pretreated with oleate may exhibit more intense hemolysis accelerating activity than untreated albumin (Fig. 3). This observation suggests dissociation of the albumin and oleate upon dilution, so that both then act to accelerate lysis. Such findings would account for the frequent ability of small quantities (0.10 ml) of normal human plasma to induce more intense oleate hemolysis acceleration than larger quantities (0.2-1.0 ml) upon addition to 3.0 ml oleate-erythrocyte systems.

TABLE II. Acceleration of Oleate Hemolysis by Unesterified Cholesterol.

2.5 ml sodium oleate* + .5 ml standardized human erythrocyte suspension +	Exp. 1	Exp. 2
Isotonic buffered saline	1.0†(1.0)	1.5 (1.5)
Unesterified cholesterol in isotonic buffered saline (1 mg/ml)	.33 (30)	.33 (35)

* Final oleate concentration = 4×10^{-4} M.

† Minimum oleate-erythrocyte contact time (min.) at 25°C after which 0.10 ml of saline or cholesterol induce immediate 100% lysis. Figures in parentheses give 100% lysis time when sequence of combining is reversed, *i.e.*, when oleate and 0.10 ml saline or cholesterol are mixed first, followed by addition of erythrocytes. All values are mean of 3 determinations.

Effect of cholesterol on oleate hemolysis. Cholesterol has been reported to be important for the fatty acid hemolysis protective properties of plasma(11). The ability of cholesterol (in isotonic buffered saline colloidal suspension) to protect against fatty acid hemolysis was confirmed during this study. Cholesterol, however, behaves similarly to whole plasma in that when added *after* human erythrocyte-oleate contact, hemolysis is accelerated, (Table II). It is unlikely, though, that plasma cholesterol accounts for the entire oleate hemolysis accelerating activity of human plasma. The oleate lysis accelerating activity of human plasma albumin (total cholesterol = 116 mg %) was similar to that of the corresponding whole plasma (total cholesterol = 234 mg %) (Table I), yet the total cholesterol level was lower. Indeed, bovine serum albumin containing the lowest cholesterol concentration (50 mg %) was the most effective oleate hemolysis accelerator. It is appreciated that these measurements are limited to total cholesterol and that only the unesterified moiety affects fatty acid hemolysis(11); nevertheless the results suggest that plasma factors other than cholesterol are also impor-

tant in lysis acceleration of oleate pretreated human erythrocytes.

Specificity of oleate hemolysis acceleration by human plasma. Although marked species variation existed in erythrocyte susceptibility to oleate lysis, plasma from rats, rabbits, guinea pigs, and sheep were all found capable of accelerating hemolysis of homologous erythrocytes pretreated with oleate. Moreover, hemolysis accelerating activity of mammalian plasma is not restricted to oleate. Sodium palmitate and stearate though less effective than sodium oleate as hemolytic agents (at pH 7.2) yielded qualitatively similar relations with respect to hemolysis protective and accelerating influences of homologous plasma. Indeed, the capacity of the same substance to protect against and to accelerate fatty acid hemolysis (depending upon the sequence of fatty acid addition) was not restricted to plasma or plasma factors. Sodium cyanide retarded hemolysis when added to oleate solutions prior to human erythrocyte insertion (7); final concentrations of 1 x 10^{-3} M sodium cyanide consistently accelerated lysis of oleate pretreated human erythrocytes.

Discussion. Since 1940, numerous studies have indicated some relation between hyperlipemia and *in vivo* hemolysis. Hyperlipemia induced by high fat diets or by intravenous infusions of lipid emulsions or hyperlipemic plasma accelerates erythrocyte destruction; at times hemoglobinemia is evident(3-5). Frank anemia may be produced by repeated intravenous fat emulsion infusions(12). Such *in vivo* observations support the hypothesis that the hyperlipemic state constitutes an important physiologic mechanism in human erythrocyte destruction(13). The precise manner whereby *in vivo* hemolysis develops during hyperlipemia, however, has been incompletely defined.

The findings presented in this report indicate that once lytic quantities of fatty acid are bound *in vitro* by human erythrocytes, homologous plasma, the plasma albumin and globulin fractions, or unesterified cholesterol (as well as cyanide) reverse their protective action and accelerate hemolysis. This acceleration can be attributed to a stoichiometric plasma interaction with the altered erythrocytes. Ability to block simultaneously and progressively both hemolysis protection and acceleration by pretreatment of plasma fractions with increasing quantities of fatty acid suggests that plasma sites which bind fatty acid and protect against fatty acid hemolysis, accelerate lysis once the fatty acid has "coated" the erythrocyte membrane. Whether plasma acts by "tearing" the fatty acid from the cell wall or by combining at the cell surface to form a new complex is undetermined; the underlying mechanism, however, appears to depend in some way upon acceleration of intrinsic fatty acid lytic activity. Changes in surface tension of the suspension medium are not responsible for fatty acid hemolysis accelerating activity of homologous plasma. The mechanism of cyanide action is unknown.

Intensity of fatty acid hemolysis acceleration by human plasma or plasma fractions is not uniform. Variations can be attributed to differences in capacity of plasma and plasma fractions to bind fatty acid and to a plasma inhibitor which can be "inactivated" by dilution. Indeed, upon sufficient dilution, the inhibitor actually enhances lysis acceleration; this inhibitor possesses the activity of unesterified plasma fatty acid.

A hypothesis linking certain aspects of hyperlipemia, *i.e.* increases in unesterified plasma fatty acid, with *in vivo* hemolysis now may be suggested from the results of the present and related previous(1,2,7) studies: Introduction into the circulation of *free* fatty acid by infusion(14), from postprandial chyle(15), from hydrolysis of circulating chylomicra(16,17), and possibly from fat depots(18), would result at the introduction site, in competitive binding by plasma and erythrocytes. Those erythrocytes binding sufficient fatty acid to exceed the hemolytic threshold may potentiate lysis by releasing lytic factors upon hemolysis during transit. The surrounding homologous plasma not alone fails to be protective once erythrocyte fatty acid binding occurs, but accelerates hemolysis. The remainder of the exogenous fatty acid, that bound by plasma albumin, globulin, and unesterified cholesterol to form unesterified plasma fatty acid, is rendered virtually inert hemolytically. *It is not the increase in plasma unesterified fatty acid*

that abruptly accelerates hemolysis during hyperlipemia—it is the exogenous free fatty acid bound by erythrocytes before plasma binding occurs. Plasma unesterified fatty acid increase is merely an indicator that exogenous fatty acid has been added to blood; it is *during* addition that conditions for maximum hemolysis acceleration are attained. Once bound by plasma, exogenous fatty acid is rapidly transferred to tissues for oxidation or esterification(19,20), probably without constituting any further serious immediate erythrocyte threat. Increased plasma unesterified fatty acid would, however, constitute an insidious threat by a) decreasing plasma ability to protect against additional free fatty acid, and b) providing erythrocytes with a source of sparingly released fatty acid.*

Whether intact chylomicra can induce hemolysis is unknown. However, since chylomicra are chiefly triglycerides(21), they can be regarded as potential *free* fatty acid reservoirs. *In vivo* hemolysis acceleration during chylomicronemia would, in part, be a function of rate of intravascular chylomicra hydrolysis. Since heparin provides optimal conditions for such intravascular hydrolysis, maximum hemolysis acceleration during chylomicronemia should develop following heparin administration. This conforms with experimental observations(6). Normally, in absence of exogenous heparin, the bulk of chylomicra hydrolysis occurs in extravascular depots(19,21,22) where a large fraction of the triglyceride fatty acid is directly oxidized without reentering the circulation(19); this process thus apparently serves as a homeostatic mechanism for mitigating intravascular hemolysis. However, in certain human and animal subjects, exogenous heparin may not be necessary for intravascular chylomicra hydrolysis since the untreated plasma contains active enzymes capable of inducing significant fatty acid release (23-25). Competitive erythrocyte-plasma binding of such released fatty acid might explain, at least in part, ability of normal chylomicra-rich plasma to induce hemolysis *in vivo* and to increase erythrocyte mechanical fragility *in vitro*(5,26).

The above interpretations permit clearer analysis of the observations of previous investigators(5,6,15,26). The proposed fatty acid-erythrocyte-plasma interactions are not specific for one fatty acid, for one blood group, or for the human. Comparable results, at least *in vitro,* were obtained with the several fatty acids tested, with all major human blood groups, and with sheep, rat, rabbit, and guinea pig erythrocytes. The suggested hypothesis linking *in vivo* hemolysis with increases in unesterified plasma fatty acid appears to possess general applicability.

Summary. 1. Pretreatment of fatty acid with human plasma, plasma albumin or globulin, unesterified cholesterol, or cyanide retards or prevents hemolytic activity. Once lytic quantities of fatty acid are bound by human erythrocytes, the above substances reverse their protective activity and accelerate hemolysis. 2. Plasma and plasma factors appear to accelerate intrinsic oleate hemolytic activity rather than act *via* a secondary superimposed lytic mechanism; the mode of cyanide action is unknown. 3. The oleate hemolysis protective and accelerating properties of human plasma can be inhibited progressively and simultaneously by pretreatment with oleate. Both mechanisms appear to depend upon plasma ability to bind fatty acid. 4. The intensity of oleate hemolysis acceleration by human plasma or plasma fractions is not uniform. Variations can be attributed to differences in fatty acid binding capacities and to inhibitors which possess unesterified fatty acid activity. 5. A hypothesis is suggested for linking increases in mammalian plasma unesterified fatty acid with *in vivo* hemolysis.

* Erythrocytes removed from humans one hour after intravenous injection of albumin-bound C^{14} labelled unesterified palmitic or oleic acid contained 1% of the radioactivity of equivalent plasma volumes; by this time plasma fatty acid radioactivity had fallen to 1% of initial values(20), so that erythrocyte fatty acid content was now only 1/10,000 of that equivalent initial plasma volumes.

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Conversion of Corticosterone-4-C^{14} to Aldosterone by Human Adrenal Slices.* (25076)

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Investigations of the biosynthetic pathways of aldosterone have yielded conflicting results. Kahnt(1) demonstrated incorporation of desoxycorticosterone-21-C^{14} into aldosterone using beef adrenal homogenates. Addition of progesterone to the incubation medium did not increase aldosterone production. Rosemberg(2) has shown that perfusion of isolated calf adrenals with progesterone increased production of aldosterone while perfusion with corticosterone or desoxycorticosterone had little or no effect. Incubation of C^{14}-labelled progesterone, desoxycorticosterone or corticosterone with beef capsule strippings by Ayres(3) and later by Travis(4) demonstrated the incorporation of each into aldosterone. The data are conflicting in regard to the most efficient precursor. Little is known of the pathways of aldosterone biosynthesis in man. Seltzer(5) demonstrated that infusion of large quantities of corticosterone increased urinary excretion of aldosterone. However, it was not determined whether this increased aldosterone excretion resulted from a direct incorporation of corticosterone or by indirect effect of corticosterone upon adrenal cortical function. Moreover, despite continuous infusion of corticosterone, aldosterone excretion diminished. The present report describes incorporation of corticosterone-4-C^{14} into aldosterone by human adrenal slices.

Methods. A patient with metastatic cancer of the breast underwent a bilateral adrenalectomy. The right adrenal was immediately immersed in cold isotonic saline, then freed of fat, weighed, and sliced with a Stadie-Riggs microtome. Approximately 2.22 x 10^5 DPM (1.4 x 10^5 CPM)/24 μg of corticosterone-4-C^{14}§

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TABLE I. Chromatographic and Isotopic Characteristics of Aldosterone.

System	Exp.	Mobility, cm/hr	$R_{cortisol}$*	Amt, μg	C^{14}, cpm	S.A., μc/mg
I Toluene propylene glycol	A	4.5/13	2.1			
	B	4.8/13	2.0			
II Bush C	A	11 / 3	1.0	3.2	4720	1.0
	B	11 / 3	1.0	2.9	8800	2.1
III E_2B	A	19 / 8	.79		1904†	
	B	18 / 8	.78		3084†	

* $R_{cortisol}$ refers to mobility of unknown compound to standard cortisol. Mobilities shown for compound isolated are similar to those of authentic aldosterone in these systems.

† 86 and 87% recovery respectively of the aliquot of aldosterone from the Bush C chromatogram which was applied to the E_2B system.

in ethanol was added to each of 4 flasks (A-1, A-2, B-1, B-2) and the ethanol evaporated. Approximately 500 mg of adrenal slices and 20 cc of Krebs-Ringer bicarbonate buffer with 200 mg % glucose were added to each flask. Incubation was carried out at 37°C in a Dubnoff incubator in an atmosphere of 95% O_2, 5% CO_2 for 2 hours. The incubation began approximately 45 minutes after surgical removal of the gland. After incubation the media of flasks A-1 and A-2 were pooled to form flask A; B-1 and B-2 to form flask B. The media were extracted twice with 3 volumes of redistilled chloroform. The chloroform extracts were washed once with 1/10 volume 0.05 NaOH, twice with 1/10 volume distilled water, dried with anhydrous sodium sulfate and evaporated *in vacuo*. The residues were applied to paper chromatogram in the toluene-propylene glycol system(6) along with appropriate standards. The steroids were located by ultraviolet scanning method. In this system, aldosterone has the same mobility as cortisone. The cortisone area was eluted with ethanol and rechromatographed in the Bush C system(7). The amount of aldosterone eluted from the Bush C system was estimated by alkaline fluorescence(8) using cortisol as the standard. An aliquot of the eluted aldosterone was rechromatographed on the E_2B system(9). The aldosterone area from Exp. B was eluted and the eluate mixed with 25 μg of aldosterone monoacetate. Acetylation was performed with 25 lambda of 15% acetic anhydride in anhydrous benzene, and 20 lambda of absolute pyridine for 48 hours at room temperature. The diacetate derivative was extracted with methylene chloride and chromatographed as indicated in Table II. Aldosterone diacetate migrates with androsterone in system IV and with Δ-4-androstene-11 β ol, 3, 17-dione in System V as reported by Kliman(10) and confirmed in this laboratory. After elution from the second diacetate system, chromic acid oxidation of radioactive aldosterone diacetate was performed. The monoacetate oxidation product was chromatographed in the cyclohexane-benzene-methanol-water system where it had the same mobility as 11 α-hydroxy progesterone.‖ Radioactivity was counted to a standard error ± 2% in a liquid phosphor scintillation counter with a counting efficiency of 64% for C^{14} and a background of approximately 20 CPM.

Results. Chromatographic mobilities from successive paper chromatographies of the "aldosterone" from Exp. A and B are listed in Table I. These mobilities are similar to those of authentic aldosterone. After elution from the Bush C chromatogram (System II), the quantity of aldosterone was approximately equal in each experiment. However, incorporation of corticosterone-4-C^{14} was appreciably greater in Exp. B. Radioactivity in the aldosterone areas of A and B represented approximately 2% and 3% respectively of the added C^{14} precursor. When aliquots of the aldosterone were rechromatographed in the E_2B system (System III) 86% and 87% of radioactivity was located in the aldosterone area

§ Corticosterone-4-C^{14} was obtained through courtesy of Endocrinology Study Section, Nat. Inst. Health (sp. act. 4.16 μC/mg.)

‖ Personal communication of Peterson, R. E., and confirmed by authors with chromic acid oxidation of authentic aldosterone diacetate-C^{14}.

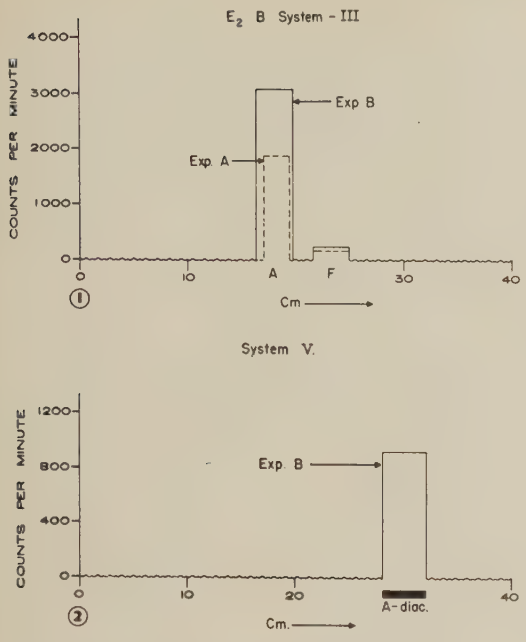

FIG. 1. Location of carbon radioactivity on paper chromatogram. After aldosterone (A) and cortisol (F) areas were cut out, remainder of chromatogram was cut into 4-5 cm strips, eluted and counted. The irregular baseline indicates background activity. 0 denotes origin.

FIG. 2. Location of carbon radioactivity on paper chromatogram. Roman numeral refers to paper chromatogram system listed in Table II. After aldosterone diacetate (A-diac.) area was cut out, remainder of chromatogram was cut into 4-5 cm strips, eluted and counted. The irregular baseline indicates background activity. 0 denotes origin.

(Fig. 1). After the aldosterone area was eluted the remainder of the chromatogram was cut up into 4-5 cm strips, eluted and counted.

An aliquot of aldosterone from Exp. B eluted from the E_2B system was mixed with 25 μg of authentic aldosterone monoacetate, acetylated, and the diacetate derivative chromatographed as indicated in Table II. After the second chromatogram (System V), 61% of radioactivity corresponded with the ultraviolet absorbing area of aldosterone diacetate. No other area contained a significant amount of radioactivity (Fig. 2).

An aliquot of the aldosterone diacetate was oxidized with chromic acid and chromatographed in System IV (Table II). Seventy % of radioactivity was located in the aldosterone monoacetate oxidation product area. The percentage recovery represents the limitation

TABLE II. Chromatographic Characteristics of Aldosterone Diacetate. 1541 cpm* 25 μg aldosterone monoacetate: acetylated.

System		Mobility, cm/hr	Rf†	C^{14}, cpm	Recovery, %
IV	Cyclohexane 4 Benzene 2 Methanol 4 Water 1	35/12	1.0		
V	Cyclohexane 4 Dioxane 4 Methanol 2 Water 1	30/10	1.0	940	61
	470 cpm: Chromic acid oxidation				
IV	Cyclohexane 4 Benzene 2 Methanol 4 Water 1	19/12	1.0‡	328	70

* Exp. B—aldosterone from E_2B system.

† Rf aldosterone diacetate refers to mobility of radioactive "aldosterone diacetate" to authentic aldosterone diacetate standard.

‡ 11α-OH progesterone was used as the reference standard.

of the oxidation method rather than impurities in the aldosterone diacetate since in the authors' hands the best recoveries with authentic aldosterone diacetate-C^{14} have been about 80%.

Discussion. Human adrenal tissue slices synthesized aldosterone from corticosterone C^{14} *in vitro*. Although the amount of aldosterone produced was small, the chemical evidence indicates the compound isolated was aldosterone. The radioactive compound had the same mobility as aldosterone in 3 successive chromatographic systems; after acetylation as aldosterone diacetate in 2 systems; and as the aldosterone monoacetate oxidation product after chromic acid oxidation.

This evidence directly demonstrates that corticosterone may be a precursor for aldosterone formation. It is supported by the indirect evidence of Seltzer(5) who showed that increased quantities of aldosterone were excreted in the urine after infusions of corticosterone. The present experiments do not exclude other pathways or imply that corticosterone is the most efficient precursor of aldosterone.

The variation of incorporation of corticosterone-4-C^{14} between Exp. A and B indicates the dangers of quantitatively comparing different precursor incorporation when tissue

slices are used. Despite the fact that approximately equal weights of adrenal tissue from the same adrenal and equal quantities of radioactive corticosterone were present, Experiment B had twice the specific activity of Exp. A, but similar microgram amounts of aldosterone. It is difficult to interpret this quantitative discrepancy.

Cell permeability, precursor pool size, and state of enzyme activity are important variables in *in vitro* steroid synthesis. For ideal comparisons of different precursors, the same adrenal tissue should be studied simultaneously, using equimolar tracer quantities of precursors with identical specific activities; and rates of synthesis of aldosterone with its immediate precursors determined.

Conclusions. Synthesis of aldosterone by human adrenal slices from corticosterone-4-C^{14} has been demonstrated *in vitro*.

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Analysis of Serum Magnesium in Presence of Calcium with Chrome Fast Blue BG. (25077)

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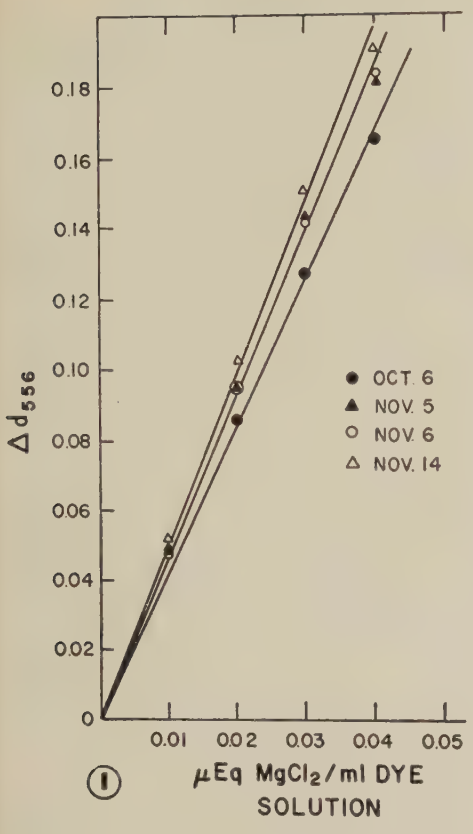
Colorimetric methods for determination of magnesium have utilized principally a series of yellow dyes(1) (Titan yellow, brilliant yellow, thiazol yellow, Clayton yellow) and Eriochrome Black T(2). Of the yellow dyes, Titan yellow has been widely accepted, but, as with all yellow dyes, a stabilizer such as gum ghatti, or polyvinyl alcohol must be used and pH values of 12.8 or more maintained. Methods using other dyes require removal of calcium and/or elapses of time for color development. Use of azo dye, 1, 8-dihydroxy-2-(3′-chloro-6′-hydroxybenzene azo) naphthalene-3-6-disulfonic acid, or Chrome Fast Blue BG, requires no removal of calcium, no waiting for color development, and no stabilizers. It is sensitive to quantities as small as 0.01 microequivalents of magnesium/ml of dye solution. This dye was first used by Japanese workers and was introduced in this country by Carson (3). Its application to analysis of magnesium was initiated in this laboratory by Lewis and Kerwin(4) and has now been refined.

Methods. Chrome Fast Blue BG was obtained from American Cyanamid Co., Bound Brook, N. J., and used after further purification.* An aqueous solution of 0.001 M (molecular weight of Na salt = 518.5) was prepared and can be stored several months. To 7 ml of 0.001 M Chrome Fast Blue BG was added 10 ml of 1 M ethylamine buffer (adjusted to pH 11.1 with HCl) and 83 ml of water. To 3 ml of this buffered dye solution in a Corex cuvette was added solutions of 0.2% trichloroacetic acid (TCA) for blanks, of $MgCl_2$ in 0.2% TCA for standardization (Fig. 1), and of the deproteinized serum (see below) for determination of Mg-content of serum. All optical densities were obtained with Beckman DU spectrophotometer. A standard

* Procedure for purification is available from Am. Cyanamid Co.



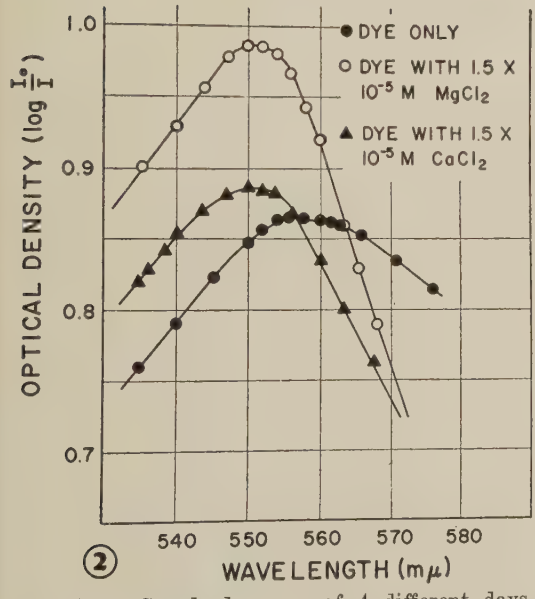


FIG. 1. Standard curves of 4 different days. $MgCl_2$ in 0.2% trichloroacetic acid.

FIG. 2. Plots of absorption spectra of Chrome Fast Blue BG with and without calcium and magnesium.

curve was made each day with buffered dye prepared for that day. Blood serum was deproteinized by either of 2 methods, the choice depending upon volume available.

Method 1. When several ml of serum were available, it was made 4% TCA by adding one volume of 20% TCA to 4 of serum. It was mixed by stirring with glass rod. The mixture was heated in water bath at 90-95°C for 5 min. and then centrifuged. To one volume of supernatant fluid was added 19 volumes of demineralized water thus making the concentration of TCA 0.2% and Mg and Ca content 1/25 of that in original serum. *Method 2.* Small volumes of serum (ca 0.1 ml) were made 4% TCA by adding equal volume of 8% TCA. This mixture was stirred to break up coagulated protein and the tube set in water at 90-95°C for 5 min after which the volume was increased to 40 times the original volume of serum with demineralized water, thus making the concentration of TCA 0.2%. The suspension was filtered through as small a circle of ashless paper as practical. Whichever method was used to deproteinize, every effort was made to remove all protein because any remaining in the supernatant solution, or filtrate, would go back into solution and bind magnesium when it was put into the alkaline dye solution. The diluted supernatant fluids were mixed for analysis by procedure given above. The difference in optical density (Δ d) between this mixture and a blank solution composed of 3 ml of buffered dye and 1 ml of 0.2% TCA was obtained at a wave length between 555 and 557 mμ (see below). Amount of magnesium in serum was ascertained from standard curve (Fig. 1) of Δ d and by calculation with proper dilution factor. This method for analyzing blood serum for magnesium without removing calcium depended upon obtaining the difference in optical density between dye with and without deproteinized serum at a wave length isosbestic (Fig. 2) for dye plus calcium and dye alone. This wave length was usually within 0.5 mμ of 556.5 mμ and was ascertained precisely for each preparation of dye by exploring between 555 and 557 mμ for that wave length which gave a Δ d value of zero, between a cuvette filled with 3 ml of dye and 1 ml of 0.2% TCA

TABLE I. Magnesium in Blood Sera of 4 Groups.

No. of determinations	Range of values (meq/l)	Mean (meq/l)
8	1.41-2.05	1.80
5	1.72-2.04	1.90
12	1.15-2.57	1.69
12	1.41-1.76	1.55

and one filled with dye plus 10^{-7} moles of $CaCl_2$ added in 1 ml of TCA. The method was tested by ascertaining amounts of magnesium that could be recovered from whole and deproteinized sera to which $MgCl_2$ was added. Preliminary tests showed that when varying amounts of $MgCl_2$ were added to whole serum, about 100% could be recovered. Two extended tests were then done in which added amounts of $MgCl_2$ were varied over the range of Fig. 1. Magnesium was added to deproteinized sera. Slopes of least square lines indicated that recovery rate in one test was 93.5 ± 4.5% and in the other 98.7 ± 3.1%.

Results. Values obtained by this method for amount of magnesium contained in blood sera are given in Table I. These sera were obtained from patients in the Clinical Center, National Institutes of Health.

Table I gives the range of individual values of magnesium in meq/l and the means of magnesium-contents of 37 sera done in 4 groups of determinations. Some ranges are large, *e.g.*, group No. 3. The lowest value of 1.15 meq/l, however, was obtained in 2 other analyses. The same applies to highest value 2.57, *i.e.*, high values were also obtained in 2 other analyses.

Mean values of magnesium in these blood sera compare well with those already in the literature. Some of these in meq/l are 1.66 (5), 1.89(6), 1.75(7), 1.58(8), and 1.80(9).

An advantage of this method is that the calcium of the specimen does not have to be removed. To make sure that calcium was not interfering, $CaCl_2$ was added to solutions used for standardization so that the Ca : Mg ratio ranged from 10:1 to 0.1:1. Such additions of calcium did not change the Δ d_{556} of buffered dye solutions containing 0.01, 0.015 and 0.02 meq of magnesium/l. Furthermore, all specimens of serum lots 3 and 4 of Table I were done with and without decalcification. Calcium was removed by making undiluted serum 0.8% ammonium oxalate. Portions of sera from which calcium was removed had mean magnesium-contents of 1.81 and 1.63 meq/l, while whole sera had 1.69 and 1.55 meq/l.

Summary. 1. A method is described for determination of magnesium in blood serum using the azo dye Chrome Fast Blue BG. 2. Use of this dye affords immediate determination of serum magnesium without removal of calcium and with no addition of stabilizers. 3. The dye as used here is sensitive to as little as .01 microequivalent of magnesium. 4. Thirty-seven sera were analyzed. These had an over-all magnesium-content of 1.73 meq/l of sera.

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Physiological Function of Alpha Cells of the Pancreas.* (25078)

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Much is known of biochemical and physiological actions of glucagon, a substance considered by most to be an alpha cell hormone of the pancreas. However, our knowledge of functions of the alpha cell based on morphological alterations is very confused. Among functions suggested are those linking it with development of diabetes(1,2), production of elastase(3,4), regulation of somatic growth (5) and fat metabolism(6). Non-diabetic dogs devoid of alpha cells can be obtained by selective partial pancreatectomy. Glucagon and alpha cells are permanently absent from remaining pancreas of these animals(7,8). The present report investigates some general metabolic aspects in such dogs devoid of alpha cells.

Materials and method. Eighteen mongrel dogs, kept in individual metabolic cages and weighed approximately twice a week, had free access to measured water and received abundant mixed diet until weight remained stationary for about 1 month. They were then fed a diet of 200 g of bread and 300 g of raw tripe for about 30 days prior to pancreatectomy. Surgical procedures were performed under Nembutal anesthesia. In 10 dogs, body and tail of pancreas were removed, the uncinate process transplanted subcutaneously and a skin pancreatic fistula created. At this time, a complete transverse section of the uncinate process proximal to body of pancreas was taken for histological examination. Because no alpha cells were found in these biopsies, it could be assumed that no alpha cells were present distal to these biopsies(7). Five dogs died of postoperative complications. Five survived experimental period. The first day after surgery, animals received only milk and sugar. From second day they were given 100 g bread and 300 g raw tripe. All dogs received 100,000 I.U. of penicillin and 5 units of Protamin Insulin Zinc for first 3 days. Blood samples were obtained from leg veins 1½ hours after morning meal and levels of glucose(9), cholesterol(10), NPN(9) and urea (9) were then determined. Glucose tolerance tests (GTT) were done as follows: glucose (0.3 g/kg in 20% solution) was injected intravenously in fasted anesthetized (Nembutal) dog within 20-30 seconds. Blood samples were taken prior to and at 30, 60 and 90 minutes after glucose injections. At least 3 GTT were done before surgery and at least one afterwards. Twenty-four hour urine specimens were collected and specific gravity and pH determined. In addition, concentrations of the following substances were determined at specified periods (Fig. 1 and 2): total nitrogen(9), urea nitrogen(9), ammonia(9), albumin(9), glucose(11), PO_4^{---}(9) and Cl^-(9). Na^+ and K^+ concentrations were determined in urine using Coleman flame photometer. Acetone was determined with Acetest, (Ames Co., USA). Dogs were killed by overdose of Nembutal at 71, 49, 59, 50, and 57 days after surgery. Samples of pituitary, thyroid, parathyroid, lung, heart, intestine, liver, pancreas, spleen, adrenal, kidney and gonads were taken for histological examinations. Search was made for any residual pancreatic fragments and all suspected tissue was examined histologically. Tissues were fixed in Zenker formol, processed according to methods previously described and a trichrome stain was used on paraffin sections 2.5 μ thick(12). Pancreas and pituitary were also stained with aldehyde fuchsin(13) and with modification of chrome-alum hematoxylin and Masson's trichrome technics(14). Glycogen was stained in pancreas, liver and kidneys with periodic acid Schiff controlled by diastase digestion. Eight untreated dogs served as morphologic

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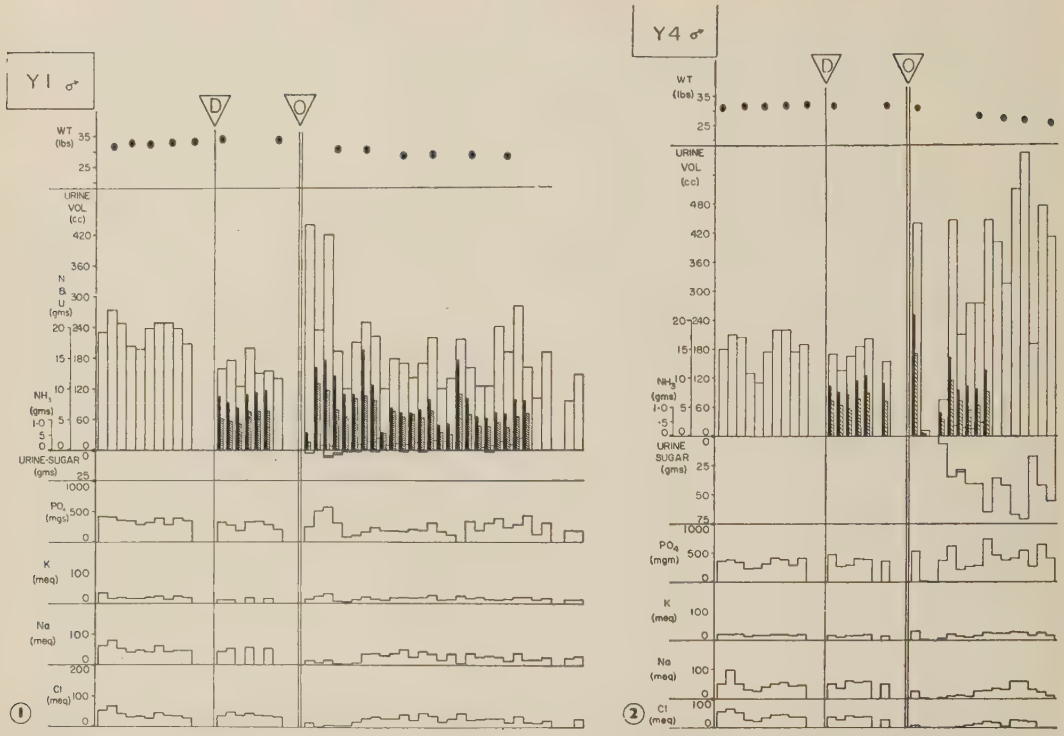

FIG. 1 and 2. Summary of data pertinent to one non-diabetic animal, Fig. 1, and one diabetic animal, Fig. 2. Determinations of urinary volume and nitrogen may be interpreted as follows:

Daily Observations

- ▭ Vol of urine/24 hr
- ▨ Urinary urea nitrogen/24 hr
- ■ Total urinary nitrogen/24 hr
- □ Urinary ammonia/24 hr

In the 269-day period before diet \D/, graphs show data for about 10 consecutive days while animals were on unrestricted food intake. In the 30-day period before operation \D/–\O/, there is another set of data for a given number of consecutive days while animals were on restricted diet. After operation \O/, a number of values were obtained on consecutive post-operative days whereas the last 2 sets of determinations, when done, were taken from 15 to 30 days later and a few days before termination of experiment.

controls only. For biochemical aspect, each operated animal served as his own control during period before operation.

Results. Before operation, the GTT were normal except for first one which usually was of subdiabetic type, probably because of initial poor nutritional state of animals. Average pre-operative blood levels in mg% were: glucose 86; cholesterol 223; urea 17; and NPN 29. Results of examining urine before operation during pre-diet and diet periods showed no significant difference (Fig. 1 and 2).

Shortly after surgery, animals lost some weight but this loss did not progress except in 2 which developed diabetes, as indicated by hyperglycemia and marked, persistent glycosuria. The other 3 dogs, while showing a diabetic type GTT, were normoglycemic and had only occasional slight glycosuria. Slight ketonuria was seen occasionally in 2. NPN increased in all dogs reaching an average of 82 mg %. There was no change in blood cholesterol and urea. During first week after surgery, there was marked reduction in urine Na^+ and Cl^- but no change or slight increase in content of K^+ and PO_4^{---}. By tenth day after operation all electrolytes had reached pre-operative levels. Except for 2 dogs with glycosuria and one, which had transient hyperglycemia, urine volume did not change appreciably. Water intake before and after

operation ranged from 50-1,000 and averaged about 250 ml. At all times urine volume was correlated closely with fluid intake. Total nitrogen and urea in urine decreased slightly for first 48-72 hours after operation but maintained normal values thereafter. The very few determinations of ammonia were within normal range. No changes were observed in pH (6.2-8.8) nor in specific gravity (1.045-1.068) of urine between pre- and post-operative periods. In a few instances, traces of albumin were found in urine of some dogs before and after pancreatectomy. No alpha cells were found in the remnant of the pancreatic transplant obtained at autopsy nor was the structure of the delta and X cells altered. Minimal degranulation of beta cells was present in 3 dogs whereas marked beta cell degranulation was present in the 2 with diabetes. No islet tissue was present in periduodenal tissues. As in previous work, no specific changes were found in the extrapancreatic tissues of these dogs devoid of alpha cells(7).

Discussion. We wished to determine what biochemical alteration, if any, would result from a complete lack of alpha cells. Except for NPN, which showed a moderate rise in all cases, and blood glucose, which increased in some animals, no significant persistent changes were found. We are not certain about the reasons for the NPN increase. However, lack of significant increase in urinary N and lack of continued weight loss, except in presence of diabetes, suggest that the blood NPN increase may be due to inhibition of protein synthesis.

Because in dogs, glucagon has a marked enhancing effect on renal excretion of Na^+, K^+, Cl^- and PO_4^{---}(15), urinary electrolytes were studied. The changes here reported are probably not due to lack of alpha cells. Similar excretion patterns have been described following surgical trauma(16); moreover, urinary electrolytes returned to normal levels within 2 weeks after operation.

An alpha cell hormone regulating fat metabolism(6) has been postulated in the rabbit on the basis of hypercholesterolemia following cobalt chloride treatment. Hypercholesterolemia was probably not due to alpha cell damage because, as is now generally accepted, cobalt-treated rabbits do not show alpha cell degeneration(17,18). Moreover, the present experiment does not support the idea that alpha cells regulate blood cholesterol levels.

Many investigators have suggested a close relationship between anterior pituitary growth hormone and alpha cells(1,5,19). Lack of changes in the pituitary in our experiment, as well as in pituitaries of dogs with experimentally altered pancreatic alpha/beta cell ratio(2,7,8), does not favor such relationships.

The diabetic aspect of some of these operated dogs is dependent upon number of beta cells left in transplanted pancreas as shown previously(2,7,8).

Lack of specific extra-pancreatic morphological alterations in dogs devoid of alpha cells is in agreement with previous work where no histological change was found that could be attributed to the absence(7), absolute decrease(8) or relative increase(2) of alpha cells. Possibly alpha cell hormone, not necessarily glucagon, acts only during period of growth. Studies are now in progress using puppies devoid of alpha cells to investigate this possibility.

Summary. 1) A study of levels of several electrolytes and various other substances concerned with protein, lipid and carbohydrate metabolism has been made in blood and urine of dogs subjected to removal of alpha cell containing part of pancreas by selective, partial pancreatectomy. 2) Despite previous suggestions by others, there was no definite evidence in our work of a relationship between functional activity of alpha cells and any of the substances studied, with the possible exception of blood non-protein nitrogen. Since this was significantly elevated in all experiments and was not associated with a significant increase in urinary nitrogen excretion nor loss of weight, it is possible that absence of alpha cells exerts a slight inhibitory effect on protein synthesis.

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Glycoprotein Content of Serum Lipoproteins.* (25079)

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There is evidence to suggest a relationship between glycoprotein content of arterial wall on the one hand and aging process and arterial disease on the other. Thus, the arterial intima and media stain increasingly for mucopolysaccharides with age and in presence of atherosclerosis(1,2), even though chemical determinations have not shown an increase in hexosamine concentration of arterial tissue with age or arterial disease(3-5). Another possible link between glycoproteins and arterial disease is provided by relationship between lipemia clearing factor, probably a heparin-containing protein(6), and lipid metabolism. An inhibitor of lipemia clearing factor containing hexosamine has also been demonstrated(7). Hexosamine and glycoprotein content of serum itself is said to increase with age and in presence of atherosclerosis(8). Finally, glycoproteins of ground substance appear to be in equilibrium with the serum(9). Since not only glycoproteins, as discussed, but also lipids play a part in processes associated with atherosclerosis and do, indeed, coexist in the lesion itself, it seemed of interest to study their interrelationship in serum from which the arterial deposits may be in part derived. Present studies were undertaken to determine glycoprotein content, expressed as hexosamine concentration, in lipoproteins of human serum. If glycoproteins and lipoproteins were linked in the serum, these substances might be simultaneously deposited in the arterial wall after infiltration. The current studies suggest that serum glycoproteins and lipoproteins are not linked to any appreciable extent. It would seem likely, therefore, that glycoproteins in the arterial wall are either deposited from serum independent of lipids, or are derived wholly or in part from local alterations in the arterial wall.

Methods. Blood was obtained from 4 healthy blood donors A, B, C, and D. Blood was allowed to clot in dry flasks and serum separated. All operations were carried out in the cold as far as possible during all stages. Lipoproteins were separated in

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† Special Research Fellow, Nat'l Heart Inst.

TABLE I. Hexosamine Concentrations in Lipoprotein Fractions of Human Serum.

	Hexosamine concentration in mg/100 ml of native serum			
Donors	A	B	C	D
Total serum	78	89	84	86
I. Chylomicrons	.84	3.04	1.68*	.05†
Total lipoproteins (α & β)			1.89	1.68
Infranates	76.5		102.2	73.5
II. Chylomicrons			5.1 *	.05†
β Lipoproteins		3.08	3.1	2.0
α "		.85	.24	.21
Infranates			69.2	77.3

* Not respun. † Identical experiment.

Spinco Model L ultracentrifuge using rotors 30 or 30.2 at approximately 79,000 x gravity or rotor 40 at 105,400 x gravity. In runs A and B, chylomicrons were not spun off separately. In runs C and D, chylomicrons were removed first after layering serum with normal sodium chloride, spinning in rotors 40 and 30.2, respectively, for 120 minutes and removing top fraction with tube slicer. Native serum in runs A and B and chylomicron infranatants in runs C and D were then adjusted to densities of 1.21 and 1.063, respectively, using the formula of Havel, Eder and Bragdon(10) for calculating requisite amounts of NaCl and KCl. The solutions were then spun in rotor 30 for 14 hours and the top fractions again separated in tube slicer, removing all lipoproteins where density of 1.21 was employed and β lipoproteins (with or without chylomicrons) where tubes were spun at density of 1.063. In the latter case, more salt was added to bring the density to 1.21 for further separation of α lipoproteins; these were again spun 14 hours in rotor 30 and top fraction removed in tube slicer. All top fractions containing lipoproteins were recentrifuged 14 hours with salt solution of requisite density using rotor 40 or 30.2, to remove lipoprotein contaminants. This was usually achieved after 2 respinnings. Respinning was carried out in each instance except in chylomicrons in run C. All infranatants in runs A, C and D were carefully collected and measured. Hexosamine was determined by Rimington's modification of Elson-Morgan procedure(11) in all lipoprotein fractions and infranatants, as well as native serum. From knowledge of hexosamine concentrations and total volumes, amounts of hexosamine could be determined in each specimen. In reporting results, all figures were converted to amount of hexosamine/100 ml of native serum. In calculating total hexosamine infranate concentrations, hexosamine in the lipoprotein respin infranates was included among the non-lipoprotein hexosamine. All final infranatant solutions were free of cholesterol as determied by the method of Abell and associates (12).

Results. The data indicate that concentration of hexosamine in lipoprotein fractions is small (Table I). The proportion of total serum hexosamine contained in lipoprotein fractions was consistently less than 5%, except in second part of run C, in which the chylomicrons had not been respun and were, no doubt, admixed with serum.

Hexosamine recoveries were calculated for 5 runs in which infranates were collected; their range was between 83 and 126% with mean of 98%. These recoveries appear not unreasonable in view of the inherent errors involved in repeated volumetric measurements and in the chemical method itself. It is concluded that no appreciable amounts of hexosamine were lost from the lipoprotein fractions during their physical separation since, in slicing the tubes, the cut was always made slightly below the line of demarkation between supra- and infranatants.

Comments. The present data differ from those recently reported by Kirby(13) who found that a major proportion of human serum mucopolysaccharides were precipitated in association with lipoproteins in Cohn frac-

tions III and IV-1. The discrepancy between her results and ours may be due to the difference in methods used. Kirby's results suggested but did not prove that there might be a linkage between lipo- and glycoproteins. Our data do not suggest existence of major linkages of this kind and seem to indicate that the glycoproteins in the arterial wall are either deposited from serum independently rather than alongside the lipoproteins or arise from local synthesis.

Oncley also believes that lipoproteins are essentially carbohydrate-free, but stresses lack of specific methods for detection of these substances in this situation(14). It is possible that even the small amount of lipoprotein-hexosamine detected in our experiments does not reflect the presence of mucopolysaccharides. Findings similar to present data have also been reported by Heide and coworkers(15).

Our results should not be taken to indicate that there is no association whatever between lipoproteins and glycoproteins in serum or tissue. While amount of glycoprotein, expressed as hexosamine associated with lipoprotein fractions was relatively small, glyco-lipid complexes may still be of importance in certain phenomena relating to lipid transport and deposition.

Summary. The amount of glycoprotein, expressed in terms of hexosamine concentration, was determined in lipoprotein fractions of 4 human sera, separated in ultracentrifuge at various densities. On an average, no more than approximately 5% of total serum hexosamine was detected in lipoprotein fractions.

Ultracentrifugations were carried out with the help of Beverley J. Neff and Gilbert Lewis, chemical determinations by E. Nagler.

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Some Compounds Active Against Experimental Visceral Leishmaniasis.* (25080)

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In exploring further usefulness of a new 8-day method for screening compounds for activity against visceral leishmaniasis(1) we have shown that cotton rats, chinchillas, and even mice can be used equally as well as golden hamsters(2). Preliminary experiments have also shown a marked activity of amphotericin B, and its more soluble form Fungizone, against this infection in hamsters (2). That polyenes might be effective against leishmanial infections was also suggested by Grossowicz and Rasooly(3) on the basis of their powerful trypanostatic effect *in vitro* against *Herpetomonas culici-*

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darum. This report details the present status of our findings with a series of active compounds, especially 2 polyenes, in *Leishmania donovani* infections in hamsters and mice.

Materials and methods. Procedures used were those of the 8-day method(1,2). Parasites of the Khartoum strain of *L. donovani*, in saline suspensions of ground spleen of an infected donor hamster, were inoculated intravascularly [intracardically (IC) for hamster; intravenously (IV) for mouse]. Treatments were given in 6 daily doses beginning one day after inoculation. Necropsies were performed on day 8, at which time liver impression smears were made and stained with Giemsa for the parasite counts. Parasite densities were expressed, as previously described(1,2), as "total" parasites in the organ. Untreated control groups were necropsied at 1-2 hours after inoculation, to confirm establishment of infection, and at 8 days. In every experiment, one group, treated with the pentavalent antimonial, sodium stibogluconate (Pentostam†), at 94 mg/k, was included for comparison. All compounds except xerosin were administered to hamsters intraperitoneally (IP) in 0.4 or 0.5 ml/100 g body weight (b.w.). Most compounds were administered to mice IP, 0.2 ml/20 g b.w. However, Fungizone was given IV, and xerosin subcutaneously, both 0.2 ml/20 g b.w. All compounds except nystatin were given in saline. Nystatin was given in an aqueous dilution of vehicle N, N-dimethylacetamide. Maximum tolerated dose of amphotericin B in hamsters IP is 300 mg/k. Maximum tolerated doses have not been determined for other compounds under these treatment procedures in these hosts.

Results. Effects of compounds tested on visceral leishmaniasis in hamster and mouse appear in Tables I and II. Uniformity of response from experiment to experiment both in untreated controls and in animals treated with Pentostam is additional evidence of the reproducibility of results by this method(1, 2). With the amount of Pentostam administered, reproduction of parasites was nearly or completely inhibited in each experiment. The variability of response is similar to that previously reported(1) and is clearly shown here in the hamster to be a function of the level of parasitization induced by inoculation. Effect of drug administration is remarkably similar in mouse and hamster in spite of species' differences in susceptibility to this parasite(4).

Amphotericin B is poorly tolerated when administered IP into hamsters, causing extensive adhesions at high dosages. Maximum effectiveness appears at the level of com-

TABLE I. Effects of Treatment on Parasite Densities in Liver of Hamsters. Infection intravascular with *Leishmania donovani*.

Animal groups	Dosage, mg/k	Trials with hamsters, parasite density in liver (as "total" parasites) 1	2	3	4
Untreated controls					
One hr after inoc.		51	12	29	38
8 days *idem*		2307	533	854	1425
Treated					
Pentostam	94	185	8	20	142
Amphotericin B	300	354			
	60	205			
Fungizone	20		3		
	5		<4	0	
	2.5			<2	
	1.25			35	
	.675			286	
Fumagillin	30		186		
	20	1470	202		
	10		209		
	2	2571*			
Xerosin	100				843
	50				851
	25				742
TWSb	200		14		
	40		46		

"Total" parasites are shown as group medians for hamsters and group means for mice. Six to 12 animals constitute a group. All groups except the first were necropsied 8 days after inoculation. All values except those marked with asterisk are significant deviations from values of 8-day controls (p values <0.05). Values marked "less than" indicate that, for more than half the animals in group, no parasites were found on liver impression smears. A portion of data in hamster trials 1, 2 and 3 has previously been reported(2).

† We are indebted to the following for compounds used: Drs. D. W. Adamson (sodium stiboglucinate, Pentostam Wellcome), E. A. H. Friedheim (antimony dimercaptosuccinate, TWSb), V. Groupé (xerosin), G. F. Otto (fumagillin, Fumadil Abbott), and Joseph N. Pagano (amphotericin B, and Fungizone Squibb and nystatin, Mycostatin Squibb).

TABLE II. Effects of Treatment on Parasite Densities in Liver of Mice. Infection intravascular with *Leishmania donovani*.

Animal groups	Dosage, mg/k	Trials with mice, parasite density in liver (as "total" parasites) 1	2
Untreated controls			
One hr after inoc.		29	32
8 days *idem*		276	363
Treated			
Pentostam	94	51	135
Fungizone	2.5	<3	
	1.25	<7	
	.675	31	
	.337		103
	.168		245
Nystatin	2		152*
	1		410*
	.2		337*
	.1		394*
Xerosin	100		120
	50		154
	25		149

For additional details, see footnote, Table I.

pound where these adhesions are minimal. In the mouse treated IV with Fungizone, no toxic effects were noted, even in the highly effective range of dosages.

Xerosin, as previously reported for virus infections(5), exerts a significant effect on parasite reproduction, but inhibition is not complete even close to the maximum tolerated dose. Fumagillin acts very much like xerosin. TWSb was active at levels near the maximum tolerated dose. Although unlike the antibiotics reported here, it is included in further evaluation of the 8-day method.

Nystatin was minimally active at the highest concentration used. As seen in Table II the numbers of parasites in the liver were not unequivocally significant from the control but the numbers of parasites in the spleen were significantly reduced over those found in the controls (1.5 *vs.* 5.6 "total" Lds, respectively; $P = 0.001$).

The activity of amphotericin B is especially noteworthy. At 5 mg/k it produced the same effect as 188 mg/k of Pentostam; namely, no visible parasites in the sample counts of spleen or liver at 8 days.

Summary. Using an eight-day method for screening compounds against experimental visceral leishmaniasis, the following compounds showed activity in hamster or mouse infection or in both: amphotericin B (and Fungizone), fumagillin, nystatin, Sb dimercaptosuccinate and xerosin. The activity of Fungizone is especially noteworthy.

The valuable assistance of Paul A. Actor and Richard N. Rossan is gratefully acknowledged.

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Effect of Prior Inoculation of Packed Erythrocytes on Survival of Skin Homografts in Rats.* (25081)

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Production of a "second-set" reaction to skin homografts in rabbits following primary inoculation of whole blood or one of its fractions has been ascribable solely to the leucocytes; no such reaction was detected in response to primary inoculations of erythrocytes(1). On the other hand, erythrocytes are capable of stimulating resistance to sarcoma homografts in mice(2). In the present study, a prior inoculation of erythrocytes

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TABLE I. Survival of Homografts on Non-Immunized and Immunized Random-Bred Rats as Determined by Epidermal Survival.

Hosts† immunized with	Post-operative day of examination										
	6	7	8	9	10	11	12	13	14	15	16
—	2/2*	1/1	4/4	1/2	4/4	2/3	2/2	1/2	1/3	0/2	0/1
Skin homograft	1/3		2/6		1/8		0/2		0/2		0/1
Red cells	1/2		1/4		2/5	1/1	0/3	0/2	0/2	0/2	0/2

* No. of grafts with surviving epidermis/No. of grafts examined.
† Donors of antigens were same individuals that furnished test homografts.

apparently brought about an earlier rejection of skin homografts in rats.

Materials and methods. Experiments were first carried out with random-bred rats and later with 3 strains of inbred rats. Full thickness grafts were transplanted by a method similar to that described by Billingham and Medawar(3). Graft survival was primarily determined from histological sections of grafts cut at 5 or 6 vertical levels. A graft was recorded as having broken down when destruction of its epidermis was apparently complete. In 2 experiments, rats were "immunized" prior to grafting with packed erythrocytes prepared as follows: Six to 8 ml of blood were withdrawn from a single donor by cardiac puncture, defibrinated by gentle agitation in a flask containing glass beads, then placed in agglutination tube with internal dimensions of 10 x 105 mm and centrifuged at approximately 1900 x g for 12 minutes. Supernatant plasma was drawn off and 0.6 - 0.8 ml of packed cells carefully withdrawn from bottom of tube. This resulted in an approximate 10-fold reduction in number of leucocytes when compared to whole blood. Each rat received one subcutaneous injection of 0.1 ml, approximately equivalent to erythrocytes present in 0.2 ml of whole blood. *Experiments with random-bred rats.* A single line of random-bred albino rats was obtained from recently established commercial colony. Three experiments were performed. (a) *Non-immunized controls.* One donor furnished a "first-set" graft to each of several recipients. After interval ranging 6 through 16 days, the grafts were removed for microscopic study. (b) *Immunized controls.* One donor was used to supply first-set grafts as before. These were left in place and 21 days later, the same donor was used to supply a second homograft to a new graft bed in each recipient. The second-set grafts were later removed for sectioning. (c) *Rats immunized with erythrocytes.* Each animal received one subcutaneous injection of packed erythrocytes followed in 21 days by a skin homograft from the red cell donor. These grafts were subsequently removed and sectioned. Hereafter, animals treated as in (b) and (c) will be referred to as "skin-immune" and "red cell-immune" respectively. In addition to a single "test" homograft, a few animals in the immunized groups also received a homograft from animal not directly related to donor or host (these will be referred to as "unrelated" homografts) and an autograft, so that 3 grafts were linearly arranged in the same graft bed. In both red cell- and skin-immune hosts, graft survival time was reduced in comparison with non-immunized controls (Table I). No surviving grafts were found beyond 11 days in the red cell-immune and 10 days in skin-immune recipients. In homografts on untreated hosts, no surviving grafts were found beyond 14th day. One autograft failed, however, the others were apparently viable. Epidermal survival time of unrelated homografts on immunized hosts approximated that of control homografts on non-immunized hosts although it may have been slightly reduced. *Experiments with inbred rats.* The methods were essentially those described above with 2 exceptions. The time interval between immunization and test grafting was shortened from 21 to 10 days in view of collateral evidence in mice(4). Prior to sacrifice of each host presence or absence of blood circulation within grafts was determined with stereoscopic microscope(5). In doubtful cases, the host was given an intravenous injection of 30% India ink in physiological saline and

TABLE II. Survival of Buffalo Skin Homografts on Non-Immunized and Immunized Fischer Hosts as Determined by Epidermal Survival and Circulation.

Fischer hosts immunized with	Post-operative day of examination								
	3	4	5	6	7	8	9	10	11
—	4/4* (0/4)	4/4 (4/4)	4/4 (4/4)	5/5 (5/5)	4/4 (4/4)	5/5 (3/5)	5/5 (0/5)	2/4 (0/4)	0/4 (0/4)
Buffalo skin homograft	5/5 (0/5)	5/5 (0/5)	3/4 (0/4)	0/5 (0/5)	1/4 (0/4)	0/5 (0/5)	0/4 (0/4)	0/4 (0/4)	0/5 (0/5)
Buffalo red cells	4/4 (0/4)	4/4 (2/4)	4/4 (1/4)	4/4 (0/4)	4/5 (0/5)	2/4 (0/4)	0/5 (0/5)	0/5 (0/5)	0/4 (0/4)

* No. of grafts with surviving epidermis/No. of grafts examined.
Observations on circulation shown in parentheses.

the grafts reexamined. Animals were derived from inbred stocks of Fischer, Buffalo, and M520 rats in their 58th, 20th, and 60th inbred generations respectively.† Fischer rats were recipients of 3 grafts; a Buffalo skin homograft, an M520 skin homograft and an autograft. On day of sacrifice, the 3 grafts on each Fischer host were examined for the presence of circulation, then removed and fixed for subsequent histological study. On an average, 4 Fischer rats were sacrificed daily from 3 through 11 days inclusive in each of 3 experiments. (a) *Non-immunized controls.* These animals received 3 simultaneous grafts whose survivals were subsequently studied. (b) *Immunized controls.* Fischer rats received a single first-set Buffalo graft that was left in place. Ten days later, each received 3 grafts as outlined above in a new graft bed. Second-set grafts were taken from the first-set graft donor. (c) *Rats immunized with erythrocytes.* This experiment was similar to preceding except that Fischer hosts were immunized with Buffalo erythrocytes instead of a first-set skin homograft.

Results. The epidermal survival of Buffalo skin homografts on red cell-immune hosts was shortened in comparison with first-set homografts, but not to the extent observed in second-set homografts (Table II). Graft survival based on circulation yielded a similar result. Circulation was present in 3 of the Buffalo grafts on red cell-immune hosts in contrast to its absence in all second-set homografts. Circulation was present in all first-set homografts examined on days 4 through 7, and absent in all grafts subsequent to day 8. The epidermis of all autografts remained viable and circulation was present in grafts examined on days 4 through 11. On non-immunized hosts, survival of M520 grafts approximated that of Buffalo homografts. On immunized hosts, however, circulation and epidermal survival continued for about a day longer in the unrelated M520 grafts than in Buffalo homografts.

Discussion. The apparent reduction in survival time of skin homografts in animals receiving prior inoculation of packed erythrocytes resembles the reported resistance against tumor homografts following red cell inoculation(2). These findings appear to be contrary to the report that red cells are unable to elicit transplantation immunity(1), however, there is some uncertainty. Decreased survival of homografts reported here could be ascribed to leucocytes present in the inocula. This is possible, but appears unlikely in view of small numbers of leucocytes present and of studies on induced resistance to a transplantable tumor in mice where it was indicated that degree of resistance was more a function of number of cells than kind of cells injected(2,6). In addition, our experiments involved a different species. Furthermore, the donor-host strain combination may be an important factor and recent evidence from mice indicates that the erythrocyte may lack certain transplantation antigens(7).

The combination of unrelated homograft and a homograft from the donor of red cells or first-set skin graft was especially useful in random-bred rats where more variation in graft survival was encountered. An immune

† Breeding stocks of inbred rats were obtained from Dr. George Jay, N.I.H., Bethesda, Md.

response could usually be detected since epidermal destruction was usually more advanced in the homograft from the red cell or first-set skin graft donor. The somewhat reduced survival time of unrelated homografts on immunized hosts may have been the result of a relatively small number of antigenic factors shared in common between the homografts(8). Autografts remained viable and apparently were not influenced by adjacent degenerating homografts. This observation parallels that of Eichwald and his co-workers(9).

Summary. Skin homografts were made between individuals of a single line of random-bred and between 3 strains of inbred rats. Graft survival was determined from histological sections of graft epidermis, and presence or absence of circulation. Survival time of skin homografts was reduced in individuals that had received a prior inoculation of packed erythrocytes.

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Isolation of Coagulation Factors by Continuous Flow Electrophoresis.* (25082)

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Continuous flow paper electrophoresis, recently(1) applied to study of blood coagulation proteins, achieved partial separation and recovery of these factors. The present studies used this technic, confirmed and extended the findings of Lewis, *et al.*(1), and provided new data on the Stuart, proconvertin, and Hageman clotting factors.

Materials and methods. The principles of continuous flow paper electrophoresis are described by Block, Durrum, and Zweig(2). The Beckman/Spinco model CP was used with barbital buffer pH 8.6, ionic strength 0.02, and 14°C. 15-20 ml samples, previously dialyzed overnight against the buffer at 4°C were fractionated by constant current of 40 milliamps which developed 500 volts. 32 fractions were collected during the usual run of 16-20 hours. At the end of this time the curtain was removed, dried 30 minutes at 110°C, and dyed with bromphenol blue. Protein in each sample was determined by a biuret method. Fibrinogen, prothrombin, proconvertin, PTC, AcG, and AHF were measured by standard assays(3). The last 2 factors, found labile under the conditions in early experiments, were excluded from the later routine. Hageman factor was estimated using Hageman deficient substrate in partial thromboplastin time test(4). Stuart factor was assayed by specific assay(5) which used a substrate deficient only in Stuart factor. *Refined preparations.* In many instances, fraction collecting tubes could be selected which contained only Stuart, proconvertin, or Hageman factor. These selected fractions were concentrated by overnight dialysis against 10% gelatin, which removed 25-40% of water and buffer salt. The dialysate was lyophilized and stored at –20°C until reconstituted with distilled water. To refine Hageman factor, the pH was adjusted to 5.2 with dilute acetic or hy-

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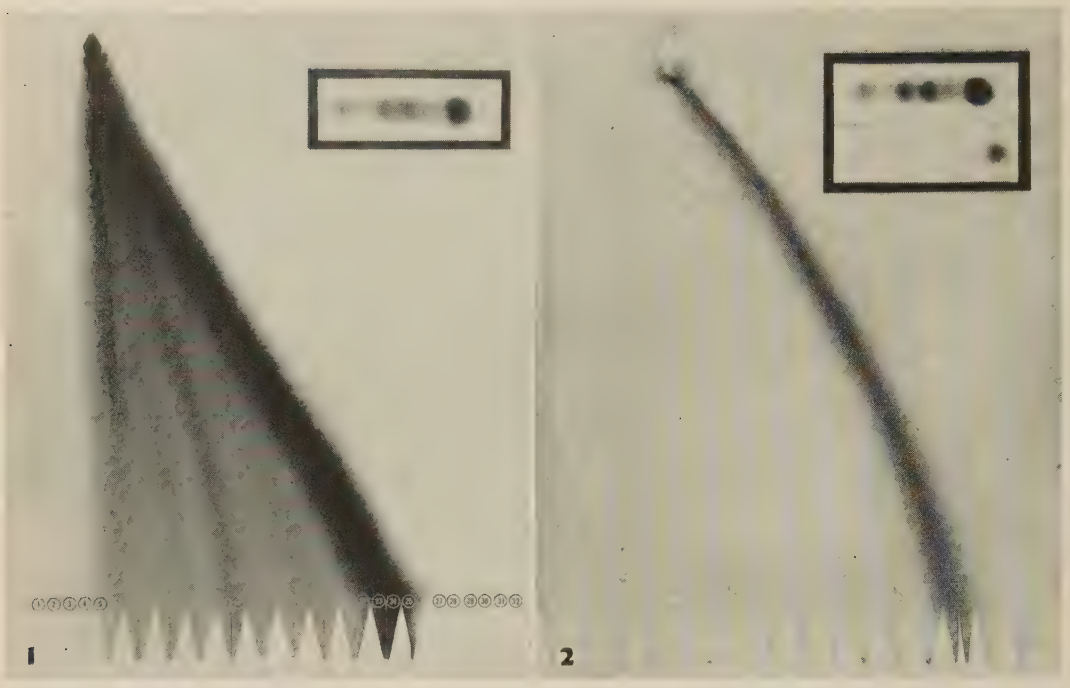

FIG. 1. Normal plasma continuous flow electrophoresis pattern with paper strip pattern (inset) for comparison. Bands, left to right, represent gamma, beta, alpha 2, alpha 1 globulins, and albumin.

FIG. 2. Continuous flow pattern of refined Stuart factor preparation. Inset shows paper strip pattern of preparation compared to normal plasma.

drochloric acid and the precipitate, recovered in barbital-saline buffer, lyophilized. For Stuart or proconvertin, the appropriate gelatin dialysate was adjusted to pH 7.0 and adsorbed with $BaSO_4$. The sediment was eluted with 0.2 M trisodium citrate, dialyzed against 0.3% NaCl until free from citrate, and then lyophilized. It is recognized that these products are not physicochemically pure. They are, however, concentrated and freed from much inactive protein and inhibitory actions of excess buffer salt. These refined preparations serve as excellent materials in studies of basic clotting factor reactions(6).

Results. Fractionation. A typical normal plasma pattern is shown in Fig. 1. For comparison, a routine paper strip pattern of the same plasma is shown in the inset. The bands correspond to usual protein zones, with albumin the darkest band on the right. The numbered tabs are shown at bottom of continuous flow pattern. In this run, tubes 5-26 contained protein. Clotting factor assays showed results similar to those of Lewis *et al.*(1), namely: incomplete resolution, with fibrinogen and Hageman in beta and gamma globulin areas; prothrombin, proconvertin, and PTC in the alpha 2, alpha 1, and late albumin areas; and AcG in the albumin area. Our specific assay showed Stuart factor in the albumin and pre-albumin areas, thus different from proconvertin. Stuart factor alone was found in tubes 25-26; these were pooled and refined further, as described above. The product was reconstituted with distilled water in 1/10 the original buffer volume and rerun on the curtain. Fig. 2 shows the resultant pattern. Stuart activity was found only at points stained by the dye. The inset in Fig. 2 shows the paper strip pattern of this preparation, compared with normal plasma. A single zone, corresponding to the curtain band, is seen, which overlaps the albumin and pre-albumin areas of the normal plasma. Thus, Stuart factor is found in the albumin and pre-albumin areas of normal plasma.

Testing of refined fractions. Table I shows assays for prothrombin (ProT), proaccelerin (AcG), proconvertin (ProC*), Stuart, and Hageman (Hag.), performed on refined preparations. The results are compared with the substrate control (0%) and normal plasma (100%). All refined preparations clearly lack prothrombin and proaccelerin. Each refined preparation contains the factor in question, essentially free from other clotting factors. In the Hageman assay, the refined Hageman preparation had activity similar to normal plasma, whereas the other products were Hageman free. The minor shortening of clotting-times in some of the other tests with the Hageman preparation is a known characteristic of excess Hageman factor(4). In the specific Stuart assay, the results with the refined preparations are negative except for Stuart, which is comparable to normal plasma. The proconvertin assay (ProC*) is the non-specific Owren method, which shows minor correction with either proconvertin or Stuart alone. In fact, the sum of the independent substrate corrections is less than 15% normal plasma concentration. However, a combination of the 2 refined preparations corrects to better than 75%. These findings have been confirmed by correction of plasmas specifically deficient in proconvertin (Proc.–) or Stuart factor (Stuart–). The results are seen in Table II. In these experiments, the assays reflect proconvertin deficiency in *3, 6, 8*; Stuart deficiency in *4, 7, 9*; a deficiency of both in *1*; and a deficiency of neither in *2, 5, 10*. Each preparation corrects the existent deficiency in that factor, *5*; *10*. A mixture of both preparations gives a normal assay, as shown previously in Table I. However, if either factor is lacking,

TABLE I. Assays of Clotting Factor Preparations. Clotting-times, sec. at 37°C.

Preparation	Assay ProT	AcG	Hag.	Stuart	ProC*
Substrate control	113.4	70.5	>600	40.4	115.0
Normal plasma	18.5	16.6	122.0	15.7	16.9
Hageman	94.5	81.5	110.4	38.0	82.0
Proconvertin	114.0	82.6	>600	38.7	45.0
Stuart	114.2	81.4	"	15.6	63.6
ProC + Stuart	115.0	"	"	15.7	19.7

* Non-specific Owren type assay.

TABLE II. Assays on Mixtures of Factor Deficient Plasmas and Refined Preparations. Clotting-times, sec. at 37°C.

	Preparation	Assay ProC*	Stuart
1.	Substrate control	105.6	46.6
2.	Normal plasma	17.4	16.4
3.	Proc.– "	58.4	20.0
4.	Proc. prep.	45.5	42.7
5.	*3* + *4*	17.0	17.8
6.	*3* + *8*	52.0	15.5
7.	Stuart– plasma	59.7	45.5
8.	Stuart prep.	71.4	15.6
9.	*7* + *4*	42.8	43.2
10.	*7* + *8*	18.0	16.2

* Non-specific Owren type assay.

an abnormal proconvertin assay (ProC.*) results. Further tests showed the proconvertin preparation to be active in our *specific* Factor VII test(5), whereas the Stuart preparation was inactive. This is additional proof that (a) the Owren technic is dependent upon both factors, and (b) complete separation of proconvertin and Stuart has been accomplished.

Discussion. The purpose of our study was to obtain individual clotting factors essentially free from others. The above tests show achievement of this goal in the case of proconvertin, Stuart, and Hageman factor. No serious consideration of concentration was raised in this part of the work. However, potencies approximating those in normal plasma were obtained for each of the refined preparations despite considerable dilution accompanying the technic.

The previous findings of Lewis and colleagues(1) regarding electrophoretic mobilities of many clotting factors have been confirmed. New information concerns the rapid migration of the Stuart factor even in advance of albumin. Others(7) have noted this in zone electrophoresis on starch gel. The present methods are only the beginnings of an approach to the production of high potency preparations suitable for physico-chemical study. In advance of this biochemical goal, they do find immediate application to studies on thrombin and thromboplastin formation *in vitro*(6).

Summary. Continuous flow paper electrophoresis has been applied to isolation of blood clotting factors. Stuart factor has been separated from proconvertin and shown to migrate

in the albumin and pre-albumin area. Data presented show factor specificity and freedom from other clotting factors in the case of proconvertin, Stuart, and Hageman preparations.

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Effect of Antispasmodic and Ganglionic Blocking Agents on Mortality Following Electroshock Convulsions in Mice.* (25083)

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Sporadic reports have appeared which indicate that certain agents produce a high incidence of post seizure mortality following electroshock convulsions in mice. Jenney(1) has shown that reserpine produces such an effect, while Berger(2) demonstrated a similar increase in post seizure mortality with benactyzine. Toman *et al.*(3) stated that an increase in post seizure mortality occurs with a variety of apparently unrelated agents and is nonspecific. Vernier and Meckelnburg (personal communication) observed that atropine, scopolamine, and other antispasmodics also increased post seizure mortality, and we found that ganglionic blocking agents were capable of eliciting the same response. Since no studies relating to the mechanism and site of action by which such drugs act have been reported, it was of interest to study the phenomena in more detail. The present studies were undertaken to determine if post seizure lethal effect of antispasmodic and ganglionic blocking agents was related to their peripheral anticholinergic activity and to determine if such agents altered the electroconvulsive threshold.

Method. Female mice of CF#1 strain were chosen to study ability of antispasmodic and ganglionic blocking drugs to increase mortality following supramaximal electroshock convulsions. The electroshock method was a modification of that described by Swinyard *et al.*(4). A supramaximal alternating current stimulus of 120 volts was passed for 0.3 second through saline-wick corneal electrodes from a Medcraft apparatus, type B_2, series 210. Care was taken to keep the wick electrodes saturated with physiological saline at all times. Five to 7 graded doses of the agents were administered intraperitoneally, in groups of 10 mice each, to establish response relating dose and mortality following maximal seizure. Time allowed for absorption of drugs before electroconvulsive shock was determined from average time required for maximal pupil dilatation (see below). The amount of drug resulting in mortality for 50% of mice tested was expressed as post seizure lethal $dose_{50}$ (P.S. LD_{50}) and was derived according to Litchfield-Wilcoxon procedure(5). To determine if antispasmodic and ganglionic blocking drugs altered the electroconvulsive threshold, mice were pretreated intraperitoneally with either 3.84 mg/kg atropine sulfate or 3.2 mg/kg mecamylamine. Control animals received saline, and 30 minutes after treatment groups

* We are indebted to J. L. Ciminera for statistical analysis applied to certain data.

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of 10 mice each were tested with stimuli of various intensities so that the proportion of mice exhibiting maximal seizure increased from 0-100%. Square wave pulses of 2 msec. duration and frequency of 59/second were delivered through saline-wick corneal electrodes for 0.3 second from an Offner type 736-A electroshock apparatus. The current strength (in ma.) to produce maximal seizures in 50% of mice (CS_{50}) was estimated from the stimulus response relationship by the Litchfield-Wilcoxon method. As a measure of peripheral activity of the antispasmodic and ganglionic blocking agents, their relative ability to dilate the mouse pupil was determined. The method for measuring pupil diameters was similar to that described by Pulewka(6) for antispasmodics and modified by Stone *et al.*(7) for ganglionic blocking drugs. Pupil diameters of mice pretreated with antispasmodics were measured 5 and 10 minutes after treatment and at 10-minute intervals thereafter until pupil diameters returned to normal. Ganglionic blocking drugs were studied in similar manner except that pupil diameter measurements were taken at 5, 10, 20, 40, and 80 minutes after administration of drugs. Five mice/dose were employed at each of several dose levels to encompass the entire dose-response relationship. Maximal observed dilatation for each animal was averaged, regardless of time interval at which it was found, and equiactive doses were estimated graphically from dose effect line relating log dose and maximal pupil dilatation. The amount corresponding to the dose that effected a dilatation to 10 micrometer units (1 unit = 0.082 mm) was chosen and designated the ED_{10}. Acute intraperitoneal toxicity was determined in female Carworth (CF#1) mice. LD_{50} values were calculated according to the method of Weil(8) and based on doses with 10 mice/level. The following drugs were employed: atropine sulfate, atropine methyl nitrate, benactyzine hydrochloride (Suavitil, Merck & Co.), chlorisondamine chloride (Ecolid, Ciba), hexamethonium diiodide, mecamylamine hydrochloride (Inversine, Merck & Co.), mepiperphenidol iodide (Darstine, Merck & Co.), the tertiary amine analogue of mepiperphenidol (1678), methantheline bromide (Banthine, Searle), propantheline bromide (Probanthine, Searle), pentolinium bitartrate (Ansolysen, Wyeth), scopolamine hydrobromide and scopolamine methiodide. All doses were in terms of the base or ion.

Results. The strain of mice (CF#1) employed normally was not susceptible to any great extent to lethal effect of electroshock convulsions, as has been previously demonstrated(9). Death following electroshock convulsions, produced in such mice by prior administration of various antispasmodic or ganglionic blocking agents (see below), occurred immediately after termination of maximal seizure. The apnea present during seizure was indefinitely prolonged, and death presumably resulted from respiratory failure. That respiratory failure was probably involved was revealed by the finding that the heart was still beating in apparently effective manner when convulsion ceased. The course of events described for these drug-related post seizure deaths was identical to that which results in those strains of mice normally susceptible to post seizure lethal effect of maximal electroshock convulsions(9) and to those that occur occasionally in the strain employed.

The several antispasmodic agents examined for ability to increase post seizure deaths are listed in Table I. Not only were atropine, scopolamine, and mepiperphenidol active in this respect, but also their corresponding methyl quaternary ammonium homologues were several times more active than the respective tertiary amine derivative. In addition, quaternary ammonium compounds, propantheline and methantheline, were also effective.

With several agents there appeared to be a real relationship between peripheral anticholinergic activity as measured by pupillary dilatation (ED_{10}) and ability to increase post seizure mortality (P.S. LD_{50}). Thus, the ED_{10} and P.S. LD_{50} values for all agents other than atropine, scopolamine, and mepiperphenidol were similar in magnitude. Larger doses of atropine and scopolamine were needed to increase post seizure mortality than was required to dilate pupils, but with mepiperphenidol the reverse was true. In spite of these exceptions, there was a significantly positive correlation between ED_{10} and P.S. LD_{50} value.

TABLE I. Relative Activity of Various Antispasmodic and Ganglionic Blocking Agents in Producing Death following Maximal Electroshock Convulsions in Mice.

Compound	Pupil dilatation		Post seizure mortality		Toxicity	
	Peak time,* min.	ED_{10},† mg/kg I.P.	P.S. LD_{50},‡ mg/kg I.P.	Slope§	LD_{50}, mg/kg I.P.	Slope§
Antispasmodic agents						
Scopolamine methiodide	30	0.014	0.033	10.0	104	1.16
" hydrobromide	30	0.026	0.62	4.5	317	1.12
Atropine methyl nitrate	30	0.05	0.07	3.0	49	1.16
" sulfate	30	0.18	0.70	4.7	187	1.16
Propantheline	10	0.22	0.23	8.1	54	1.10
Methantheline	10	0.38	0.48	6.3	61	1.12
Mepiperphenidol	10	0.95	0.14	8.7	101	1.17
Benactyzine	5	1.8	1.3	2.9	92	1.22
1678‖	10	2.6	3.0	8.0	171	1.12
Ganglionic blocking agents						
Chlorisondamine	30	0.05	0.064	1.9	38	1.39
Pentolinium	10	0.33	0.21	3.7	36	1.11
Mecamylamine	30	1.3	1.4	2.7	39	1.15
Hexamethonium	10	10.2	2.0	2.7	42	1.22

* Time after administration at which maximal dilatation of pupil was observed. This time was allowed for absorption prior to determining the P.S. LD_{50}.

† The dose required to produce pupil diameter of 10 μmeter units (each unit = 0.082 mm).

‡ Post seizure LD_{50} and is the dose estimated to induce 50% mortality following maximal electroshock seizures.

§ The fold change in dosage necessary to give unit stand. dev. change in response. See Litchfield and Wilcoxon(5).

‖ The tertiary amine counterpart of mepiperphenidol.

Correlation coefficient was calculated to be +0.68 (P, <0.05 with 7 degrees of freedom).

For each antispasmodic the slope of dose-response line relating per cent post seizure mortality and dose was extremely flat as compared to that ordinarily obtained in determining an LD_{50}. The slopes, in terms of fold change in dose required to effect a unit standard deviation change in response(5) for P.S. LD_{50} values, varied from 3 to 10 (Table I). Similar values obtained from LD_{50} data were considerably smaller (Table I). The characteristically flat slope obtained with P.S. LD_{50} determinations suggests that several mechanisms are involved with respect to post seizure lethal effect.

An interesting biphasic action occurred with benactyzine (Table II). Low doses, that is, those in median range with respect to dilating the pupil (see ED_{10}, Table I), produced an increase of post seizure deaths. As the dose was raised, the post seizure mortality effect disappeared and was correlated with appearance of anticonvulsant effect as indicated by absence of hind leg extensor component. Ability of benactyzine to increase post seizure mortality has been reported, although the self-limiting action was not mentioned(2).

Pretreatment of mice with ganglionic blocking drugs also resulted in immediate death following maximal seizure from electroshock-induced convulsions. As with antispasmodic drugs described, there appeared to be a close relation between ability of drugs such as chlorisondamine, mecamylamine, and pentolinium to dilate the pupil and to increase mortality following electroshock (Table I). Hexame-

TABLE II. Anticonvulsant and Post Seizure Lethal Effect of Benactyzine.

Dose,* mg/kg I.P.	Max seizure,† No. protected per 10 tested	Mortality, No. dying per 10 tested
.25	0	2
.5	0	0
1.	0	3
2.	0	7
4.	0	9
8.	0	4
16.	3	1
32.	9	0

* Administered 5 min. before electroshock.

† All mice were shocked as described in text. Protection from maximal seizure was assessed by absence of hind limb extensor component.

TABLE III. Effect of Atropine and Mecamylamine on Electroconvulsive Threshold in Mice.

Stimulus intensity, ma	Atropine sulfate (3.84 mg/kg I.P.)				Mecamylamine (3.2 mg/kg I.P.)			
	Maximal seizure, No. w.s.*/10 tested		Mortality, No. dying/10 tested		Maximal seizure, No. w.s.*/10 tested		Mortality, No. dying/10 tested	
	Control group	Drug group	Control group	Drug group	Control group	Drug group	Control group	Drug group
4.2	0	1	0	0	0	0	0	0
5.0	3	3	0	2	1	0	0	0
6.0	7	6	1	4	5	5	1	3
7.2	9	9	0	8	7	6	1	5
8.4	10	9	0	8	8	9	0	7
10.0					10	10	1	8
CS_{50}, ma†	5.5	5.7			6.3	6.5		

* w.s. = with maximal seizure. All mice were shocked as described in text.
† Stimulus intensity required to produce maximal seizure in 50% of mice; calculated after Litchfield and Wilcoxon(5).

thonium, on the other hand, was relatively more effective in producing post seizure deaths than in dilating the pupil.

In comparison with antispasmodics, the slope of dose-response lines for ganglionic blocking drugs varied only from about 2-4; nevertheless, these values were still higher than the slope functions obtained in various LD_{50} determinations (Table I). Ganglionic blocking agents, like the several antispasmodics, caused death in electroshocked mice in doses much below their lethal levels when used alone (Table I).

The influence of atropine sulfate and mecamylamine on electroconvulsive threshold was determined in mice pretreated with a dose of drug which had resulted in 90% mortality following seizures. The convulsive threshold was not altered by either atropine or mecamylamine as reflected in the almost identical CS_{50} values obtained for control and treated groups (Table III). On the other hand, there was a significant increase in post seizure mortality among treated animals that displayed maximal seizures.

Discussion. Our study reveals that antispasmodic substances and ganglionic blocking agents are highly effective in producing death following electroshock convulsions in mice. The immediate cause of death appeared to be due to respiratory failure insofar as shocked mice did not resume breathing following termination of maximal seizure. The mechanism involved in this failure was not revealed, but evidently it is not due to increase in sensitivity to the shocking stimulus. At least, no change in electroconvulsive threshold was demonstrated. The data also indicate that agents employed produced an increase in seizure mortality by a site of action outside the central nervous system. This concept is supported by close correspondence between doses required to elicit post seizure death with those required to produce typical anticholinergic action of drugs studied. Moreover, it is to be noted that some of the more potent agents were onium derivatives of nitrogen, a type of compound not well distributed within the central nervous system.

The biphasic action observed with benactyzine was of interest, since it suggested that anticonvulsant substances would protect against joint lethal action of antispasmodics or ganglionic blocking agents and electroshock convulsion. This was also shown by finding that effective anticonvulsant doses of diphenylhydantoin would prevent post seizure deaths to either atropine or mecamylamine (Torchiana and Stone, unpublished). The self antagonizing action of benactyzine and that of diphenylhydantoin indicates that death occurred only if a maximal seizure was produced. A similar relationship was observed in those strains of mice normally susceptible to lethal action of maximal seizure (9).

Effectiveness of antispasmodics and ganglionic blocking agents in increasing mortality after maximal electroshock seizures probably varies with species. Atropine has been studied in rats(10) and guinea pigs(11) with respect to its effect on electroconvulsive threshold

with no mention of death occurring in either species. Atropine is also commonly employed in man as pretreatment to electroconvulsive therapy. Certain studies(12-15) provide good evidence that when used in doses sufficient to produce peripheral anticholinergic effects, the combination of atropine and electroshock did not result in fatal outcome.

Ability of antispasmodics and ganglionic blocking agents to increase post seizure mortality resembles a similar action of reserpine, epinephrine, chlorpromazine, mescaline, azacyclonol, pipradrol and dibenamine(1,3). Little information bearing on their effectiveness in this respect is available. Such data would be helpful in determining whether post seizure lethal action of this diverse group of substances is nonspecific or whether a single property or effect underlies their action.

Summary. Ability of several antispasmodic and ganglionic blocking substances to produce immediate death following maximal electroshock convulsions in mice has been demonstrated. With most agents studied (atropine, atropine methyl nitrate, benactyzine, mepiperphenidol, methantheline, propantheline, scopolamine, scopolamine methiodide, mecamylamine, chlorisondamine, pentolinium and hexamethonium), the post seizure lethal effect was produced in doses approximating that required to produce a peripheral anticholinergic effect in the same species (pupil dilatation). Neither atropine nor mecamylamine altered the electroconvulsive threshold.

Respiratory failure appeared to be the cause of drug-induced post seizure deaths, but the mechanism of action was not revealed.

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Solubility of Hemoglobin as Red Cell Marker in Irradiated Mouse Chimeras. (25084)

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Transplantation and growth of donor-type progenitors of mouse erythrocytes in lethally irradiated mice may be demonstrated by serological markers(1) when donor and recipient red cells are of different *H-2* genotypes, and by agar(2) and starch gel electrophoresis(3) when their hemoglobins give different patterns on electrophoresis. This report describes differences in solubility of various mouse hemoglobins that can be used to identify erythrocytes of genetically different types probably not distinguishable from one another serologically or electrophoretically.

* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

Materials and methods. Blood from de-

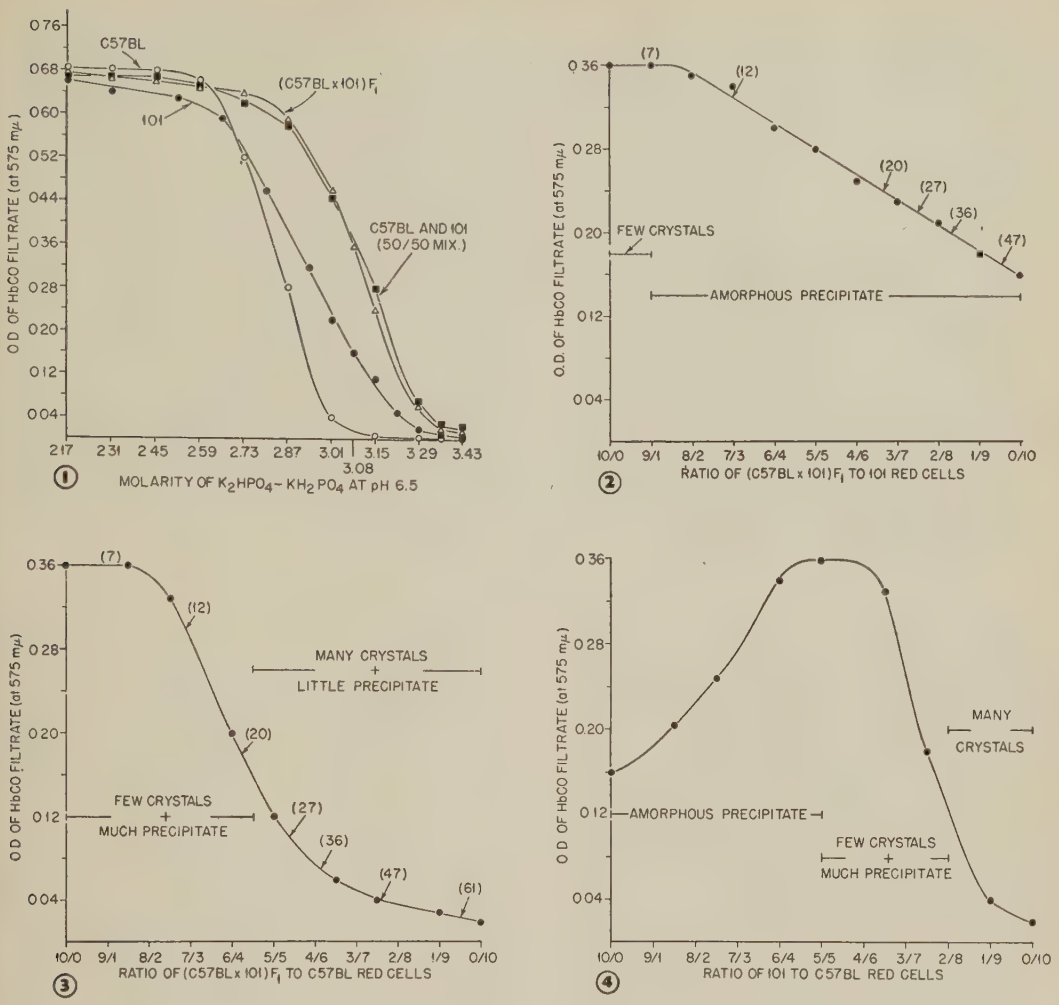

FIG. 1. Salting out curves for genetically different mouse hemoglobins. Preparations contained 0.1 ml of HbCO in total volume of 5 ml, distilled water added as necessary to change molarity of phosphate buffer. Readings were made at 21 hr (readings made prior to 18 hr were slightly higher and those after 24 hr slightly lower owing to incomplete salting out and gradual denaturation).

FIG. 2. HbCO solubility in irradiated (C57BL × 101)F_1 mice receiving transplants of 101 bone marrow. Arrows show avg O.D. found and numbers in parentheses show days after irradiation on which chimeras were tested. Data were obtained from 6 of 10 surviving lethally irradiated recipients receiving 15 × 10^6 donor bone marrow cells.

FIG. 3. HbCO solubility in irradiated (C57BL × 101)F_1 mice receiving transplants of C57BL bone marrow. For complete explanation of notations see footnote in Fig. 2. Results from 10 lethally irradiated recipients receiving 15 × 10^6 donor bone marrow cells, only 6 of which survived through day 36.

FIG. 4. Solubility of mixtures of 101 and C57BL HbCO. Shape of this curve clearly indicates that equilibrium solubility is not reached under conditions used; however, these conditions are preferred because of rapid denaturation of labile mouse hemoglobin.

capitated mice was suspended in about 200 vol of cold physiological saline. Red cells were concentrated by centrifugation, the saline was siphoned off, and the cells were washed 3 more times, each time cells were resuspended in 10 vol of saline. Packed erythrocytes were then lysed in an equal volume of distilled water, and red cell membranes were removed by centrifugation. The supernatant oxyhemoglobin (HbO_2) was converted to carbon monoxyhemoglobin (HbCO) by bubbling CO through the solution until the deep red color of HbO_2

TABLE I. Comparison of Hemoglobin of Various Mouse Strains as Regards Genotype, Electrophoretic Pattern, Solubility, and Type of Precipitate Formed.

Strain*	Genotype†	Electrophoretic pattern‡	Solubility (O.D.)§	Precipitate‖
C57BL C57L C57BR pc^{ch} 11G/Rl	Hb^1 Hb^1	Single	.01–.02	Crystalline
101 AK C3H DBA/2 RF (101 × C3H)F_1	Hb^2 Hb^2	Diffuse	.14–.16	Amorphous
(C57BL × 101)F_1 (C57BL × DBA/2)F_1 (C57L × A)F_1	Hb^1 Hb^2	"	.33–.36	Mixed¶

* Minimum of 6 mice checked for each group.
† After Gluecksohn-Waelsch (see Russell, E. S., Gerald, P. S., *Science*, 1958, v128, 1569).
‡ Nomenclature of Gluecksohn-Waelsch *et al.*(5).
§ Avg O.D. of HbCO at 575 mμ (see text).
‖ See Fig. 5.
¶ Crystals in (C57BL × 101)F_1 more numerous at higher concentrations of phosphate buffer.

became the brilliant crimson color of HbCO. Derrien's technics(4) were used to determine the solubility of HbCO in K_2HPO_4-KH_2PO_4 buffer at pH 6.5. Salting out preparations stood for 18-24 hours at room temperature before they were filtered through Whatman No. 1 filter paper. Optical density (O.D.) of the filtrates was read at 575 mμ in a Coleman Jr. Spectrophotometer, Model 6A. Precipitate formations were examined under a microscope at 430 X.

Results. Fig. 1 shows the salting-out curves for HbCO prepared from erythrocytes of C57BL, 101, and (C57BL X 101)F_1 mice. Hemoglobins of various other strains exhibited a similar relation between solubility and electrophoretic mobility (Table I). Comparable results were obtained when a few drops of whole blood from tail vein were placed in distilled water and the solution was bubbled with CO before phosphate buffer was added. Mixtures of similar types of hemoglobins [*e.g.*, C3H + 101, C57L + C57BL, or (C57BL X 101)F_1 + (C57BL X DBA/2)F_1] had solubilities characteristic of individual types being mixed, but mixtures of different HbCO types gave O.D. readings intermediate between those of original HbCO samples, increased, or decreased, depending on relative percentages of the two HbCO types in the mixture (Fig. 2, 3, and 4). On salting out, HbCO of the homozygous-diffuse type formed an amorphous precipitate, that of the single-type formed large hexagonal crystals, and that of the heterozygous-diffuse type formed a mixture of amorphous precipitate and crystals (Fig. 5). Crystals also developed in mixtures of single and homozygous-diffuse HbCO when more than 50% of the HbCO was of single-type. Optical densities of known mixtures of HbCO filtrates were measured, and precipitates were examined to determine how solubility and precipitate formation would correlate with changes in erythrocyte population during establishment of red cell chimerism. The results obtained from lethally irradiated (C57BL X 101)F_1 mice that received injections of either C57BL or 101 bone marrow cells are compared in Figs. 2 and 3 with values obtained from known HbCO mixtures. Comparative analyses of erythrocytes of chimeras with both starch gel electrophoresis and hemoglobin solubility indicated that the results of the 2 technics agreed with one another (Table II).

Discussion. The O.D. of the HbCO filtrate is an expression of solubility of HbCO at a specific pH and salt concentration. Since K_2HPO_4–KH_2PO_4 at pH 6.5 has a large buffering capacity, pH of the solution remains essentially constant as HbCO is salted out. Small differences in salt concentration, how-

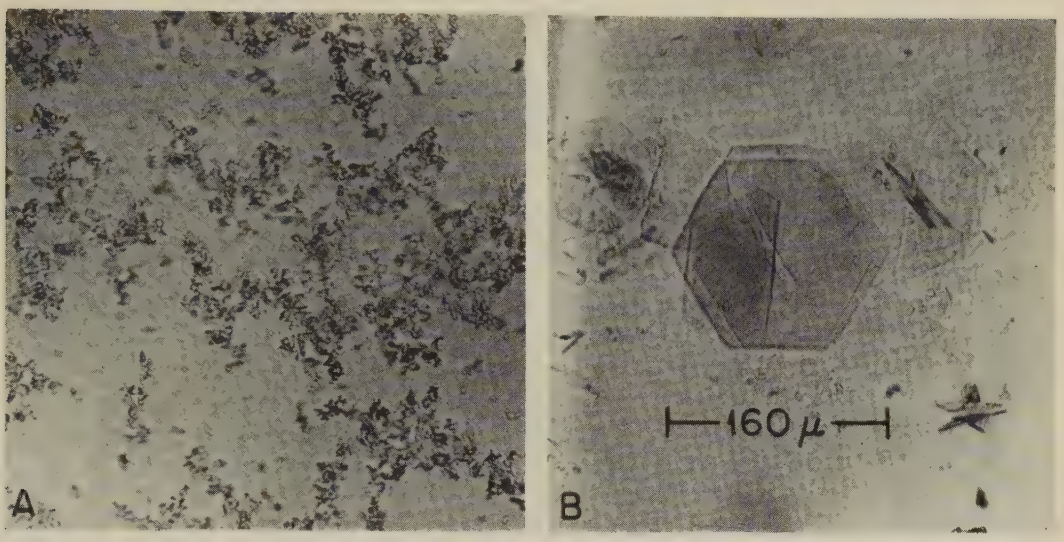

FIG. 5. A. Amorphous precipitate obtained with 101 HbCO; B. crystals formed with C57BL HbCO. Crystals were formed in 2.87 M phosphate, at which molarity size and definition seems to be maximal.

ever, cause significant changes in HbCO solubility (Fig. 1). The quantity of HbCO used should be sufficient to give final O.D. readings of at least 0.50 in low salt concentrations. Initial O.D. values of our preparations were 0.90-1.00 and final readings were about 0.65-0.70 after the preparations had stood for 18-24 hours at room temperature in 1.75 M phosphate buffer. Although under these conditions HbCO apparently did not salt out, some nonspecific denaturation of labile mouse hemoglobin nevertheless occurred. HbCO was used because it was more stable than HbO_2. If large concentrations of HbCO were used, a longer time was required to salt out the excess HbCO. Filtrates of supersaturated HbCO solutions became turbid upon standing, owing to further precipitation of HbCO. Results were more reproducible when initial concentrations of HbCO were similar, perhaps because of more-uniform gradual nonspecific denaturation as well as salting out of mouse HbCO. Quantitative determinations of the mixtures of red cells in chimeras were made with about 0.1 ml of whole blood from the severed tail vein. Blood from anemic animals frequently gave readings lower than expected since initial concentration of HbCO was less than optimal.

Possible uses of hemoglobin as a red cell marker, particularly where *H-2* markers cannot be used, have been discussed(3). The use of hemoglobin solubility is even more adaptable than electrophoresis for assay of erythrocytes in irradiated mouse chimeras since all 3 hemoglobin genotypes can be identified by their solubility differences (Table II). For example, although presence of strain 101 red cells in group B and absence of such cells in group C cannot be determined by electrophoresis, they can be readily determined by HbCO solubility. The percentage of cells of one type in mixed populations of erythrocytes can also be estimated as well by solubility as by our previously reported electrophoretic technic(3). A major drawback in the quantitation, however, is that similar optical densities from 0.16-0.36 are obtained for 2 very different populations of red cells when mice with single and homozygous-diffuse hemoglobin types are used as donors and recipients of bone marrow (Fig. 4). In this situation, the correct choice can be ascertained only by examining the precipitate or by noting the type of red cells in the populatiin that is increasing on successive weekly readings.

The genetic basis for occurrence of more than one hemoglobin in the mouse seems to be similar to that in man(5). The similarity of salting-out curves for heterozygous-diffuse

TABLE II. Identification of Hemoglobin in Irradiated Mouse Chimeras.

Group	No. of recipient mice*	Dose (r)	Donor nucleated cells ($\times 10^6$) Bone marrow† C57BL	Bone marrow† 101	Spleen‡ C57BL	Spleen‡ 101	Post-treatment (mo)	Chimera hemoglobin — Electrophoretic mobility§	Chimera hemoglobin — Optical density‖
A	2	900	15				3	Single	.039 ± .011
	5	500			120		3	"	
B	16	900		15			2–3	Diffuse	.18 ± .008
	1	600				120	3	"	
C	13	400–600			120		2–4	"	.40 ± .012
	10	"				30	2–3	"	
	3	600				12	2	"	

* (C57BL × 101)F_1; 12-16 wk old. † Inj. intrav. ‡ Inj. intraper. § As determined by starch gel electrophoresis, nomenclature of Gluecksohn-Waelsch *et al.*(5). ‖ Avg O.D. ± S.E. of mouse HbCO filtrate (see text).

HbCO and for equal mixtures of single with homozygous-diffuse HbCO (Fig. 1) suggests that each hemoglobin gene expresses itself in hybrids by controlling the synthesis of its own hemoglobin type. The development of crystals of single-type hemoglobin in mixtures of HbCO of different genotypes (see Figs. 2, 3, 4) is consistent with this hypothesis. In this respect the action of the hemoglobin genes of the mouse is similar to that of genes governing hemoglobin synthesis in man(6,7).

Summary. Genetically different mouse hemoglobins differ in solubility and crystal formation. Differences in hemoglobin solubility and crystal formation can therefore be used to identify homologous erythrocytes in irradiated mouse chimeras if the hemoglobin types of donor and recipient mice are distinguishable by these properties to begin with.

We wish to acknowledge technical assistance of D. E. Reynolds.

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Effect of Dietary Zinc Deficiency of Chicken Hepatic Cells. (25085)

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O'Dell and Savage(1,2) reported that growth of chicks was stimulated when as little as 6.6 ppm of zinc was added to a purified diet of glucose-isolated soybean protein (Drackett Assay Protein C-1) type even though the basal contained about 50 ppm of Zn. Further reports concerned with zinc requirements of chicks and poults fed highly refined diets have been reported by Norris *et al.*(3), Morrison and Sarett(4), Pensack *et al.*(5), Norris and Zeigler(6), Roberson and Schaible(7) and Supplee *et al.*(8). This communication reports histopathological findings observed in chicks fed a diet deficient in zinc 14 days following hatching.

Materials and methods. Crossbred male

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TABLE I. Size of Nucleoli of Hepatic Cells as Related to Supplemental Zinc, in 20 Birds.

No supplemented zinc Nucleoli (μ)		40 ppm supplemented zinc Nucleoli (μ)	
R-1	3.80	R-1	2.29
	4.04		2.41
	3.82		2.20
	4.01		2.24
	3.79		2.20
	3.89		2.27
R-2	4.02	R-2	2.23
	3.96		1.97
	3.81		2.03
	4.58		2.35
	3.93		2.43
	4.06		2.20
Avg	3.97	Avg	2.23

chicks from a mating of New Hampshire males to Columbian females were fed experimental diets in metal batteries, coated with plastic resin. A purified basal diet (Drackett Assay Protein C-1-Cerelose) was used. Feed and distilled water in glass containers were offered *ad lib.* for 14 days. The basal diet contained 13 ppm of zinc. It was supplemented with 40 ppm of zinc from zinc carbonate. Five chicks from each of 2 replicates were examined for histopathologic changes. Portions of heart, spleen, liver, kidney and small intestine were collected and fixed in 2% acetated, 10% formalin, Bouin's and Carnoy's solution. Tissues were processed by paraffin method in autotechnicon and stained with Harris' hematoxylin and eosin.

Results. Histopathologic observations. Only liver tissue presented histopathologic changes that could be related to dietary treatments. Enlargement of nucleoli of hepatic cells of zinc deficient birds was apparent upon casual observation of slides and this was confirmed by quantitative measurements. No differences were seen in size of nuclei between treatments.

Hepatic cells of zinc adequate chicks appeared normal while those of zinc deficient chicks appeared to have nucleoli almost twice as large as nucleoli in zinc supplemented chicks. The nucleoplasm of hepatic cells in zinc deficient birds appeared normal in other respects.

Normal and abnormal hepatic cells were observed in all slides but in varying proportions. To measure quantitatively diet induced differences in size of nucleoli of hepatic cells, 100 nucleoli were measured on each coded slide, selected at random. The method used to measure quantitative differences is similar to that employed by Jungherr *et al.*(9). There were 10 slides or a total of 1000 nucleoli measured/lot. A Spencer micrometer and 60X Bausch and Lomb objective were used. Nuclear components were considered circular and the largest diameter at sharp focus representative of size. Quantitative data significant at 0.1% level supported preliminary observations. Nucleoli of zinc adequate chicks had a mean diameter of 2.23 μ and those of zinc deficient chicks 3.97 μ or 78% larger.

Distributions of nucleolar sizes are shown in Table I.

The differences between zinc deficient and supplemented lots were considered pronounced in regard to size of nucleoli of hepatic cells.

Jungherr *et al.*(9) were the first to report nucleolar hypertrophy of hepatic cells of the chicken as a result of a nutritional factor; namely, an arginine deficient diet. They reported enlarged nucleoli in hepatic cells of arginine deficient chicks and observed that the alteration was uncommon in routine avian necropsy material and in Exp. A, D, and E hypovitaminosis of chickens. Nucleolar hypertrophy of hepatic cells was observed in rats fed toxic doses of thioacetamide(10) and in regenerating hepatic cells of rats that had been subjected to partial hepatectomy(11).

Summary. Hepatic cells of chicks fed a purified basal diet consisting of isolated soybean protein (Drackett Assay Protein C-1) with no supplemental zinc, exhibited nucleoli 78% larger than nucleoli of hepatic cells in equal number of chicks fed the same diet supplemented with 40 ppm of zinc. No other histopathologic changes were observed in sections of heart, spleen, kidney and small intestine.

We are indebted to Dr. E. L. Jungherr for suggestions in regard to quantitative differences in nucleoli of hepatic cells and use of his method for making measurements.

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Reaction of An Aspergillus Polysaccharide with Cryptococcus Capsules and Various Acidic Polysaccharides.* (25086)

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Recently, a polygalactosamine (galactosaminan) has been isolated from cultures of *Aspergillus parasiticus* by electromigration(1,2). This polysaccharide contains only galactosamine, 35% of which is acetylated(2), and when a solution of the polysaccharide is exposed to an electric current in an electrophoresis or electrodialysis apparatus it moves toward the cathode, presumably due to the positive charge conferred by non-acetylated galactosamine. The capsular polysaccharide of *Cryptococcus neoformans* moves to the anode under similar conditions due to its content of glucuronic acid(3,4). That the first of these polysaccharides possesses positively charged amino groups led to the prediction that it might react with the carboxyl groups of the *C. neoformans* polysaccharide, or, for that matter other acidic polysaccharides. Such a reaction might result in precipitation if both polysaccharides were in solution when mixed or in a "capsular reaction"(5-7) if the acidic polysaccharide were in the form of a capsule surrounding a microorganism. Such reactions occurred and a preliminary study of the interaction is presented here.

Materials and methods. Samples of polygalactosamine (APS) from *Aspergillus parasiticus*(2) were furnished by Dr. Saul Roseman, Univ. of Michigan. Stock solutions were prepared by dissolving APS (1 mg/ml) in M/100 HCl or M/20 H_3PO_4. Dilutions of the stock were made in water, saline solution, or buffer. Capsular polysaccharides of pneumococcus types 2 and 3 (S2 and S3) were isolated from broth culture as described in(8). Commercial (USP) grade gum acacia was used without further purification. Samples of dextran were kindly furnished by Dr. Allene Jeanes. Cryptococcus polysaccharides were precipitated with ethanol in presence of acetic acid and sodium acetate(4,9). Samples of Type A polysaccharide (SA) used were further purified by electromigration in an electrodialysis chamber(4). Each of the 3 cells contained 100 ml of veronal buffer pH 8.6 or acetate buffer pH 5.0 (0.025M). In addition, the center cell contained SA dissolved in buffer at a concentration of 1 mg/ml. A current of 100 m.a. was applied for 5 to 6 hours and then the SA was deposited in a gelatinous layer on the anode membrane and was scraped off and dissolved in water for final ethanol precipitation. For precipitin tests, all polysaccharides were dissolved in 0.9% sodium chloride solution. Preliminary tests indicated that good precipitation of acidic polysaccharides could be obtained with as little as 50 μg APS/ml; therefore 0.5 ml APS (100 μg/ml) was added to each 10 x 75 mm test tube followed by 0.5 ml of acidic polysaccharide or dextran at various concentrations. Tests were read after

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TABLE I. APS Capsular Reaction and Agglutination of *C. neoformans* in Saline Solution.

Reaction	*C. neoformans* cells	APS (μg/ml) 50	25	12.5	6.2	3.1	1.5	.8
Capsular reaction*	CN- 6	CR	CR	(CR)	(CR)	(—)	—	—
	15	CR	CR	(CR)	(CR)	(—)	—	—
Agglutination†	6	+	+	+	+	±	±	—
	15	+	+	+	+	±	±	—

Strength and variability of capsular reaction is indicated in decreasing order by symbols CR, (CR), (—).

* Read after 30 min. at room temperature.

† Read after additional 18 hr in refrigerator.

2 hours at room temperature and stored at 0-4°C for 4 days at which time a final reading was made. For capsular reactions, cells of *C. neoformans*, Type A, strain 6, or Type B, strain 15 were grown 2-4 days in neopeptone broth with constant agitation(4). The cells were washed 3 times by centrifugation and suspended in water, saline or buffer (10^7 cells/ml). For the test, equal volumes of cell suspension and APS dissolved in appropriate vehicle were added to small test tubes. After 15-30 minutes at room temperature and again after 18-24 hours refrigeration a drop was removed from each tube, transferred to microscope slide and examined under cover glass at 440x.

Results. When mixtures of *C. neoformans* cells and APS were examined under the microscope a pronounced clarification of the capsular outline was seen in contrast to control cells in water or saline where no capsule was visible. When electrolyte was present, this resembled the "capsular reaction" obtained with specific antibody(4-7); however, if the reactants were in distilled water, a marked contraction of the capsule occurred in response to APS. In this case, the capsule had an irregularly crenated margin and was contracted so that it was sometimes hard to differentiate from the cell wall. In either water or saline, easily discernible reactions were obtained with 50 μg APS/ml although at this concentration not all capsules reacted to the same degree. Results of titration in serial 2-fold dilutions of APS in saline solution are presented in Table I. Some reaction was obtained with as little as 3.1 μg/ml after 30 minutes incubation at room temperature; however at this concentration reactions were weak and variable and many capsules did not react at all. Additional storage of mixtures in the refrigerator for 18 hours resulted in some intensification of the capsular reaction and in agglutination of cells. At higher APS concentrations, the agglutinated cells were firmly adherent to the bottom and sides of the test tube.

Before setting up the capsular reaction, cryptococcal cells were washed several times by centrifugation. The advisability of this procedure is demonstrated in Table II where it is shown that addition of an excess of dissolved polysaccharide (SA) to cryptococcal Type A cell suspensions before exposure to APS resulted in inhibition of capsular reaction. This was apparently due to competition between the soluble SA and the intact capsule for APS, and demonstrates identity of SA with the capsular substance. It should be noted that in this experiment the final APS concentration was held constant at 50 μg/ml. Use of a smaller amount would conceivably result in more striking inhibition. It can also be seen from Table II that the capsular reaction and its inhibition by excess SA occur over a fairly wide range of pH; more extensive studies on the effect of pH are in progress.

When APS was mixed with certain polysaccharides, precipitation resulted. Typical re-

TABLE II. Inhibition of APS*— Cryptococcus Capsular Reaction by Excess SA at Various pH Values.

Buffer (M/20)	SA conc. (μg/ml) 0	1000	100	10
HCl-KCl pH 2	CR	—	(CR)	CR
Citrate pH 4	"	—	—	"
Phosphate pH 6	"	—	—	"
Veronal pH 8	"	—	—	"

CR = capsular reaction. (CR) = weak capsular reaction.

* APS conc. = 50 μg/ml.

TABLE III. Precipitation of Various Polysaccharides by APS.

Polysaccharide	Polysaccharide conc. (μg/ml) 1000	500	250	125	62	31
Cryptococcus SA	C	C	CP	P	P	(P)
Dextran	—	—	—	—	—	—
Pneumococcus S2	P	P	P	P	P	(P)
" S3	C	CP	P	P	(C)	—
Gum acacia	P	P	P	P	C	(C)

C = cloudy, CP = cloudy + precipitation, P = precipitation, ( ) = weaker reaction.

sults are presented in Table III. Precipitation occurred with all acidic polysaccharides tested, including Cryptococcus SA, Pneumococcus Types 2 and 3, and gum acacia. Although not shown in the Table, Cryptococcus SB and SC also precipitated with APS. Dextran, the only non-acidic polysaccharide tested, failed to react.

Discussion. The combination of APS, the polygalactosamine produced by *A. parasiticus,* with microbial capsules and dissolved polysaccharides introduces another interesting form of microbial interaction.

It is not yet known whether the combination of APS with microbial cells would inhibit their growth; however, this would seem to be a possible application of practical value. Combination of APS with the microbial cell surface might also serve to enhance phagocytosis of the cell. The reaction bears a superficial resemblance to the antigen-antibody reaction and may be useful as a model in studying certain aspects of the antigen-antibody combination. It also recalls the non-specific combination of proteins with microbial capsules(7) except that the APS reaction seems less dependent on pH. The APS reaction might also be of value in studying such intriguing interactions as that reported between 2 mating types of yeast by Brock(10). The APS reaction serves to emphasize that antigen-antibody-like combinations can result from the reaction of an "antigen" with a compound that bears only a superficial resemblance to an antibody; such interactions occurring among various polysaccharides, proteins and complexes within the human body may conceivably give rise to certain diseases of unknown or obscure etiology about which it is currently popular to speculate on the etiologic role of an antigen-antibody reaction.

Summary. When polygalactosamine (APS) produced by *Aspergillus parasiticus* was mixed with cells of *Cryptococcus neoformans* it produced a "capsular reaction" and agglutination similar to that caused by specific antibody. The positively charged APS molecule also caused precipitation of various negatively charged (acidic) polysaccharides including gum acacia and capsular polysaccharides of *C. neoformans* and pneumococcus. Dextran was not precipitated by APS.

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Tissue and Serum Aldolase of Rats with Primary Hepatoma. (25087)

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Aldolase, one of 5 enzymes involved in glycolysis, is normally present in tissues, cells, and blood serum of rats. It catalyses the reversible cleavage of one mole of fructose-1,6-diphosphate into one mole each of glyceraldehyde phosphate and dihydroxyacetone phosphate. Meyerhof and Lohman characterized this enzyme(1), and reported its wide distribution in animal tissues. Warburg and Christian (2) showed that aldolase and triose isomerase were consistently elevated in blood-serum of rats bearing large Jensen sarcomas. Sibley and Lehninger(3) found elevated aldolase values in blood-serum of patients suffering from various neoplastic diseases. They also showed that surgical removal of the tumor or its therapeutic regression lowered serum aldolase values to near normal levels. This paper deals with aldolase levels of tissues and serums of rats bearing a primary hepatoma and a transplanted sarcoma, with emphasis on relative aldolase level of serums from blood entering and leaving tumor tissue.

Materials and methods. Rats of Osborne-Mendel strain were used. Number of animals in each case is given in Table I. A primary hepatoma and a transplanted sarcoma were the tumors used. *A primary hepatoma.* Male and female rats 3 to 4 months old were fed a semisynthetic diet containing 0.075% paradimethylaminobenzene - 1 - azo - 2 - naphthalene for 230 days and then a laboratory diet of Purina chow pellets. Induction of these tumors and their histopathology have been described(4). Rats had large (30 to 100 g) hepatomas. The hepatoma tissue was selected by its glistening pinkish gray color and firm texture. Necrotic areas of the hepatoma were selected by the soft texture and dull brownish gray color. Non-tumorous liver tissue was taken from areas without any pale gray tumor nodules, as determined by examination with hand lens. *A transplantable sarcoma.* Pieces of spindle cell sarcoma(5) were implanted with a trochar, under the skin of right flank of male rats about 3 months old. Animals with transplants 6 cm or more in diameter and 80 g or more in weight were included in the sarcoma experiments. Method of induction of this sarcoma and histopathology of the primary tumor and transplants have been described(5). Sarcoma tissue was selected by its pearly gray color and firm texture. Necrotic areas of sarcoma were selected by the dark brown color and soft spongy texture. *Controls* included healthy, male and female rats 4 to 12 months old, weighing 150 to 400 g. Liver tissue and blood samples from hepatic vein, inferior vena cava and heart were obtained for aldolase determinations. Blood samples from hepatoma-rats were collected from the portal vein where it enters the liver, and the inferior vena cava where the hepatic veins empty into it, and those from sarcoma-rats were collected from a tumor vein and the heart. All blood samples were obtained under ether anesthesia. Serum was separated soon after clot was formed and stored in cold until analyzed. Serum samples with any trace of hemolysis were discarded. For tissue assays, rats were killed and bled, and the organs removed and placed on ice. All tissue pieces for aldolase determination were selected on the basis of gross appearance. This gross diagnosis was later confirmed by microscopic examination of adjacent piece of tissue. Two thin slices, similar in appearance, were removed from each piece of tissue. One slice was processed for microscopic examination and the second was weighed, ground in all glass homogenizer, and diluted to appropriate volume with ice-cold water. All assays were performed as soon as possible after collection of samples. Aldolase was determined by Sibley and Lehninger method(6). In all assays of tissue extracts, 0.5 ml of 5% egg albumin was added to the incubation mixture as a protective agent. A unit of aldolase is defined as amount of enzyme in unit weight (1 g) or unit volume (1 ml) of sample which can split 1 μl of fructose-1, 6-diphosphate substrate, at pH 8.6, in one hour at 38°C.

TABLE I. Serum and Tissue Aldolase Values of Normal and Tumor-Bearing Rats.

Tissue	No. of rats	Range*	Avg*
Normal control rats			
Liver	27	7,900–22,100	14,400
Portal vein serum	22	50–100	70
Vena caval "	22	"	"
Heart blood "	22	"	"
Hepatoma-bearing rats			
Hepatoma	40	8,900–20,200	12,400
Liver (non-tumorous)	30	6,900–15,400	9,600
Portal vein serum	18	200–700	375
Vena caval "	18	250–900	540
Hepatoma–necrotic	8	300–500	385
Sarcoma-bearing rats			
Sarcoma	28	5,600–10,300	7,700
Liver	28	6,800–12,400	9,100
Heart blood serum	15	140–250	176
Tumor vein "	15	130–260	194
Sarcoma–necrotic	10	150–220	178

* Units of aldolase/g of tissue or ml of serum.

Results. Serum Aldolase. Table I presents aldolase values for hepatoma and sarcoma, and for serum and liver of normal and tumor-bearing rats. In control rats, serum aldolase values of blood from portal vein, inferior vena cava and heart ranged from 50 to 100, average 70 units. Fluctuations in serum aldolase values showed no correlation with age, sex, or weight of rats studied. In rats bearing transplants of sarcoma, serum aldolase values for tumor-vein blood was significantly* higher than that for heart blood of the same rat. Average mean difference of these values was 18 with P value less than .0001. In a few rats of the hepatoma series killed before hepatomas were palpable, no significant changes were found in either serum or liver aldolase values from those in control rats. In rats bearing primary hepatomas, the average serum aldolase value of vena cava blood was 540 and that of portal vein blood was 375 units. Serum aldolase values for blood leaving the tumorous liver were consistently and significantly higher than values for blood entering the same liver. A comparison of representative serum aldolase values on individual rats is graphically shown in Fig. 1.

Tissue aldolase. Liver aldolase values for normal rats ranged from 7,900 to 22,100, average 14,400 units. The aldolase values for non-tumorous livers of tumor-bearing animals, as a rule, were lower than those for livers of normal animals (Table I). Only 11% of normal control rats, but 63% of rats bearing hepatomas and 79% of rats bearing sarcomas had liver aldolase values under 10,000 units. The range of liver aldolase values of rats bearing hepatomas was 6,900 to 15,400 units and that of rats bearing sarcomas was 6,800 to 12,400 units. Aldolase values for hepatomas ranged from 8,900 to 20,200 average 12,400 units. Aldolase values for necrotic areas of these tumors ranged from 300 to 500 units and were similar to serum aldolase values. As a rule, hepatoma aldolase values were higher than liver aldolase values of the same rat (Fig. 2). Aldolase values of sarcomas ranged from 5,600 to 10,300 with average of 7,700

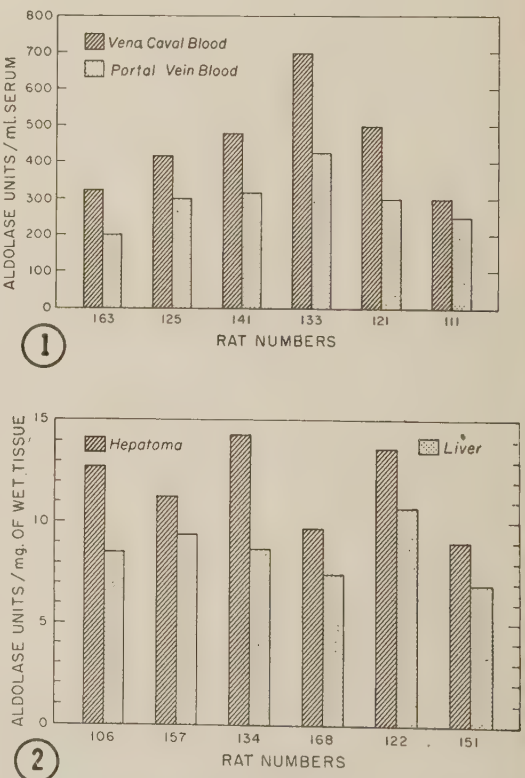

FIG. 1. Serum aldolase values for blood entering and leaving tumorous liver of 6 typical hepatoma-bearing rats.

FIG. 2. Comparison of aldolase values of hepatoma tissue with hepatic tissue (non-tumorous) of the same rat.

* All significant differences had a P value of less than .0001. I am grateful to Mr. N. Mantel of the Biometrics Branch for statistical analysis of figures.

units. About 70% of sarcomas had values from 6,000 to 9,000 units. Necrotic areas of tumor tissue had aldolase values of 150 to 220 units and were similar to serum aldolase values of the respective animals.

Discussion. This study confirms previous reports of high serum aldolase levels in rats bearing large tumors. Tumors used were carcinogen-induced primary hepatomas and transplanted sarcomas from carcinogen-induced tumors originating in this laboratory. Serum aldolase values of hepatoma-bearing rats were 5 to 8 times and those of sarcoma-bearing rats 2 to 3 times as high as corresponding values for control rats.

In rats on a carcinogenic diet, serum aldolase values were not significantly higher than those for control rats, before the hepatomas were readily palpable, and there was no significant difference between portal vein and vena cava blood values. In rats bearing primary hepatomas, the serum aldolase value of blood leaving tumor area was 20 to 65% higher than that of blood entering this area. Serum aldolase on rats with transplanted sarcoma showed similar but smaller differences between tumor-vein and heart blood. Sibley and co-workers(7) reported similar differences in serum aldolase values on blood from tumor-vein and that from the heart.

The level of serum aldolase showed a positive relationship to level of tumor aldolase. Hepatoma-bearing rats with high hepatoma aldolase levels (average 12,400 units) had high serum aldolase levels (average 375 and 540 units); while sarcoma-bearing rats with lower sarcoma aldolase levels (average, 7,700 units) had lower serum aldolase levels (average 176 and 194 units). Necrotic areas of tumors had aldolase values of the same order as serum values of animals bearing those tumors. There was no apparent relationship between amount of tumor necrosis and level of serum aldolase.

Aldolase, a normal constituent of animal tissues and cells, is constantly released into the blood stream. It is continuously removed from the blood stream(7) either by excretion or destruction. Thus level of serum aldolase is a dynamic balance between rates of its entry into and exit from the blood stream. Aronson and Volk(8) reported a rise in serum aldolase in dogs subjected to sectioning of femoral nerve or removal of entire cauda equina root system. This showed that the rate of release of aldolase by non-neoplastic tissue can be increased under certain conditions. Tumor slices, in absence of glucose in media, liberated significantly more aldolase under aerobic conditions than liver slices under similar circumstances(7). Hiatt(9) produced evidence of complete conversion of intraperitoneally injected glucose to lactate by Ehrlich ascites tumor cells, before it reached the blood stream. Horecker and Hiatt(10) attribute this unrestrained glycolysis by tumor cell to its rapid growth and consequent rapid conversion of adenosine triphosphate to adenosine diphosphate.

From above observations, it is clear that given the proper conditions, neoplastic as well as non-neoplastic cells, accelerate release of aldolase into blood stream. Thus, it is suggested that the relatively rapid growth pattern of neoplastic tissue creates conditions conducive to high glycolytic rate. This induces local cellular hypoglycemia, which accelerates the rate of aldolase release. Accelerated release of aldolase from tumor tissue may account for higher serum adolase levels in animals bearing tumors. However, the possibility of accelerated release of aldolase from other sources is not ruled out.

Summary. 1) Tissue and serum aldolase of normal and tumor-bearing (primary hepatoma and transplanted sarcoma) Osborne-Mendel rats were studied. Blood for aldolase assay was drawn from portal vein and inferior vena cava of hepatoma-bearing and control rats, from tumor vein and heart of sarcoma-bearing rats and also from heart of control rats. 2) Control rats had an average serum aldolase value of 70 units each for portal vein, vena cava and heart blood. Serum aldolase values of sarcoma-bearing rats were about 3 times and those of hepatoma-bearing rats about 8 times the values for control rats. These values averaged 375 units for blood entering tumorous liver and 540 units for blood leaving it. Serum aldolase values for heart blood averaged 176 while that for sarcoma-vein blood of the same rats averaged 194

units. Average aldolase value for normal liver, hepatoma, hepatoma-free liver, sarcoma and sarcoma-rat liver were 14,400, 12,400, 9,600, 7,700 and 9,100 units, respectively. Liver aldolase levels for tumor-bearing rats were about 35% lower than those for control rats. Aldolase values for necrotic areas of tumors were similar to serum aldolase values for respective rats.

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6-Methylmercaptopurine: Identification as Metabolite of 6-Mercaptopurine *in vivo* and Its Activity *in vitro*.*† (25088)

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6-Mercaptopurine possesses definite antitumor activity in animals(3) and is widely used clinically in leukemia(4). Previous studies demonstrated the thiol methylation of thiouracil(5) and its iodo analogue *in vivo* (6). It appeared of interest to determine whether 6-mercaptopurine can also undergo thiol methylation *in vivo* and to quantitate the extent of this reaction. 6-Mercaptopurine can inhibit incorporation of C^{14}-precursors into nucleic acid fractions of tumor cells *in vivo*(7) and *in vitro*(8). To determine whether thiol-methylation interferes with or increases this action, the inhibitory effects of 6-methylmercaptopurine (6-MeMP) and 6-mercaptopurine (6-MP) on incorporation of formate $-C^{14}$ and glycine $-C^{14}$ into nucleic acid and protein fractions of ascites tumor cells were compared.

Methods. 6-MP-S^{35}, unlabeled 6-MeMP, and 6-MeMP-S^{35} used were prepared by methods of Elion *et al.*(9,10) and their purity determined chromatographically prior to use. *Identification and quantitation of 6-MeMP in urine.* Sprague-Dawley rats weighing 250-300 g were injected subcutaneously with 48.5 mg of either unlabeled 6-MP or 6-MP-S^{35} (0.62 mc/mM) dissolved in slightly alkaline aqueous solution. The animals were placed in individual metabolism cages and urine was collected for 24 hours under toluene. Urine from animals injected with unlabeled 6-MP was lyophilized to dryness and extracted with absolute ethanol. Aliquots of alcohol extracts were then chromatographed on Whatman No. 40 paper, alongside authentic 6-MeMP, by the descending method. 6-MeMP was located by its vivid yellow fluorescence under ultraviolet light (Mineralite lamp). R_f values of authentic and suspected 6-MeMP corresponded exactly when these fractions were eluted and rechromatographed in 3 different solvent systems. The solvent systems used were n-butanol:acetic acid:water (35.5:9.6:16.0); n-butanol saturated with 2N NH_4OH; and 70% ethanol. R_f values obtained were 0.84, 0.52 and 0.81 respectively. The yellow fluorescent areas were eluted from the paper with 0.1 N HCl and their absorption spectra were compared in a Beckman U.V. spectrophotometer. Spectra of authentic and sus-

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† Preliminary reports have been presented(1,2).

pected 6-MeMP at pH 1 were identical, with the same maxima at 224 and 295 mμ. To quantitate urinary excretion of 6-MeMP, unlabeled carrier 6-MeMP was added to urine collected from animals injected with 6-MP-S^{35}. Aliquots of this urine were chromatographed on paper and the 6-MeMP was located by methods described above. To obtain the relative per cent of excreted 6-MeMP, paper chromatograms were passed under an automatically recording scanner (Actigraph) to locate the S^{35} components. S^{35} was determined by cutting out each radioactive area of the paper and counting on aluminum planchets in a gas flow counter. In 2 experiments, 0.5-1.0% of excreted S^{35} was 6-MeMP in urine collected during 24 hours after administration of 6-MP-S^{35}.

Incorporation of glycine-2-C^{14} and formate-C^{14} into nucleic acid and protein fractions of ascites tumor cells in vitro. Formate-C^{14} (2mc/mM) and glycine-2-C^{14} (1 mc/mM) were obtained from Nuclear-Chicago; 6-MP, from Burroughs Wellcome and Co.; 6-MeMP was synthesized by methods described above. Ascites tumor cells were obtained from Swiss mice inoculated intraperitoneally 6-7 days previously with Sarcoma 180 ascites tumor cells. The cells were withdrawn under light ether anesthesia, centrifuged, and resuspended in 4 volumes of clot-free ascitic fluid. To each 25 ml Erlenmeyer flask was added 1 ml of cell suspension; 1 ml of isotonic saline: 0.1 M Na_2HPO_4 buffer mixture at pH 7.4 containing 80 mg% glucose and 5 μc of either formate-C^{14} or glycine-2-C^{14}; and 0.4 ml of slightly alkaline saline containing either 6-MP or 6-MeMP, to produce a final concentration of thiopurine inhibitor of 2.5 mM. To isolate the combined nucleic acid and protein fractions, 3 ml of 95% ethanol was added to each flask at end of incubation period. The entire contents of flasks, together with 1 ml ethanol rinse, were transferred to centrifuge tubes. These were centrifuged and precipitates obtained washed successively with absolute ethanol-ether mixture and ether, then air dried. The dry precipitates were extracted with 10% NaCl at 100°C for 30 min, then centrifuged. Supernatants contained combined sodium nucleate fraction, and insoluble precipitates contained the protein fraction. Sodium nucleates were precipitated from supernatants with 2.5 volumes of absolute ethanol, redissolved in 1 ml distilled water and reprecipitated with ethanol, washed successively with absolute ethanol and ether, then air dried. Protein precipitates were washed successively with hot 10% NaCl, absolute ethanol, and ether, then air dried. C^{14}-activity of combined sodium nucleate fractions and protein fractions was determined by suspending the dried fractions in aqueous solution and plating on aluminum planchets. The planchets were dried, counted in end-window gas flow counter, and corrected for self-absorption.

The *results* shown in Table I reveal that, on an equimolar basis, 6-MeMP inhibited incorporation of either formate-C^{14} or glycine-C^{14} into total nucleic acid and protein fractions of Sarcoma-180 ascites tumor cells to a considerably greater extent than did 6-MP. It is also observed that the effect of 6-MP on formate and glycine incorporation into proteins is considerably less than its effect on incorporation into nucleic acids. This differential effect is not seen with 6-MeMP.

Metabolism and chemical stability of 6-MP and 6-MeMP in vitro. To ascertain whether tumor cells can metabolize the thio-purine inhibitors *in vitro,* ascites tumor cells were incubated with those agents under our conditions, with the exceptions that 6-MP-S^{35} and 6-MeMP-S^{35} were substituted for their unlabeled analogues and the formate-C^{14} or glycine-2-C^{14} was omitted. Stability of each inhibitor purine was determined by simultaneously incubating control flasks containing 6-MP-S^{35} or 6-MeMP-S^{35} with ascites tumor cells previously killed by boiling. At end of incubation period, 0.2 ml of 26% perchloric acid was added to each flask and the precipitated material transferred to tubes and centrifuged. Precipitates were reextracted with cold 2% perchloric acid and recentrifuged. Combined perchloric acid supernatants were neutralized in an ice bath with 0.5 N KOH and the precipitates formed were separated by centrifugation. Aliquots of clear supernatants were chromatographed on paper in 2 solvent systems (n-butanol : acetic acid : water,

TABLE I. Comparative Effects of 6-Mercaptopurine and 6-Methylmercaptopurine on Incorporation of C^{14} into Components of Sarcoma 180 Ascites Cells *In Vitro.*

Labeled compound	Inhibitor*	Protein, cpm/mg	Inhibition, %	Combined nucleic acids, cpm/mg	Inhibition, %
Formate-C^{14}	Control	22,800		2174	
"	6-MP	16,750	26.5	1159	46.6
"	6-MeMP	9,725	57.3	706	68.0
Glycine-2-C^{14}	Control	37,000		1149	
"	6-MP	33,400	9.7	871	24.0
"	6-MeMP	14,200	61.6	381	66.8

* Inhibitor conc. 2.5 mM. Values are means of quadruplicate determinations.

35.5 : 9.6 : 16.0, and n-butanol saturated with 2N NH_4OH).

Examination of chromatograms of acid soluble extracts derived from both types of incubations by scanning with the Actigraph did not reveal presence of any S^{35} compounds other than 6-MP-S^{35} and the 6-MeMP-S^{35} originally added.

Discussion. Identification of 6-MeMP as a metabolite of 6-MP and previously reported evidence for biological methylation of thiol compounds, suggest that methylation may be a generalized reaction for bio-transformation of thiol drugs *in vivo*. It would be of interest to know whether 6-thioguanine, structurally closely related to 6-MP and reported as a potent inhibitor of Sarcoma-180(11), can also undergo thio-methylation *in vivo*.

The small quantity of 6-MeMP in 24 hour urine collections appears to indicate that the magnitude of purine thiol methylation occurring *in vivo* is small. The significance of the S-methylation reaction is unknown. It may represent a detoxication mechanism, unrelated to tumor inhibitory effects *in vivo*.

That 6-MP-S^{35} did not undergo spontaneous decomposition and that it was not metabolized during incubation with ascites cells *in vitro,* indicates that its lesser effectiveness in inhibiting incorporation of formate-C^{14} and glycine-C^{14} into Sarcoma-180 tumor fractions, when compared to equimolar concentration of 6-MeMP, cannot be attributed to these factors. Methylation of a small fraction of 6-MP by ascites cells during incubation *in vitro* cannot be excluded by the analytical technics used. Therefore, it is possible that inhibitory activity of 6-MP observed, might be dependent on its conversion to 6-MeMP. However, the converse hypothesis, that activity of 6-MeMP might be due to demethylation of a small fraction to 6-MP is considered very improbable based on data shown in Table I.

From the standpoint of a structure-activity relationship, these *in vitro* data are consistent with the conclusion that inhibitory activity can be exerted in the presence of the methyl thioether linkage (CH_3-S-) and is not dependent on a free sulfhydryl group. Similar evidence has been presented by Skinner *et al.* (12). In their experiments, using inhibition of regeneration of hydra tentacles *in vitro* as assay of cell division, increase in aliphatic chain length of 6-substituted-thio-purines caused a definite increase in activity, when compared to 6-MP.

Several reports demonstrated that 6-MeMP, while possessing some degree of *in vivo* activity, is not as active as 6-MP against several mouse tumors and human leukemia(11,13,14). Two possible explanations for the apparent discrepancies between inhibitory effects of 6-MeMP *in vitro* and *in vivo* are: a. Inhibition of glycine-C^{14} and formate-C^{14} incorporation into Sarcoma-180 ascites tumor cells *in vitro* may bear no relation to antitumor effect *in vivo*. b. Differences in metabolic fates for 6-MP and 6-MeMP may exist *in vivo*. In this regard, Clarke *et al.*(11) suggested that 6-MeMP may have a metabolic fate different from that of 6-MP, due to its lack of oxidation by xanthine oxidase.

Conclusions. 1. 6-methylmercaptopurine was identified as a urinary metabolite of 6-mercaptopurine in normal Sprague-Dawley rats. 2. In *in vitro* experiments, 6-MeMP exerted greater inhibition of both formate and glycine incorporation into the combined nucleic acid and protein fractions of Sarcoma 180 ascites tumor cells than did 6-MP.

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Implantation of Normal Blood-Forming Tissue in Genetically Anemic Mice, Without X-irradiation of Host.* (25089)

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Numerous experiments(1-7) have demonstrated that blood-forming tissue may be successfully transplanted into heavily X-irradiated normal mice, but this phenomenon has never been demonstrated with adult untreated hosts. Transfers of blood-forming tissue between normal donors and genetically anemic hosts, reported here, indicate that, at least under certain circumstances, implantation of blood-forming tissue from another individual may be achieved without X-irradiation of recipient. The successfully implanted hosts include not only adult anemic mice similar to those in which implantation of isologous normal hematopoietic cells following mild (200r) whole-body radiation has already been reported(8,9), but also juvenile hosts of this genotype (WW^v) and of the lethally anemic genotype (WW). The anemia encountered in both of these genotypes has been reported (10) as a normochromic, macrocytic type. Although experiments using heavily irradiated normal hosts have demonstrated successful implantation of both homologous and isologous hematopoietic cells, homologous normal blood-forming cells have not been effectively implanted in adult anemic hosts after 200 r and much heavier radiation doses(11). These mice are extremely radiosensitive with median lethal doses in range of 200 r to 300 r(8). The low frequency of spontaneous survival to adulthood observed both in WW^v mice (25-31%) and in WW mice (0-10%)(12) make it especially doubtful whether juvenile animals of these genotypes could withstand heavy doses of radiation even as a prelude to tissue implantation. Thus, radiation to induce acceptance of blood-forming tissue appears unsuitable as therapy for these genetically anemic mice. It seemed advisable, therefore, to test for the possibility of implantation of isologous normal cells without radiation of the host. A critical test for success of implantation is possible in these genetically anemic hosts, since erythrocytes derived from normal blood-forming tissues, isologous with

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TABLE I. Success and Duration of Implants of Isologous Normal (*ww*) Embryo Liver Hematopoietic Cells in Non-Radiated Anemic Hosts.

Donor-host combination	No.	Surviving	Successful	Duration (days)
Adult hosts, WW^v (20/24)				
C57BL I.V. into WK × C57BL	3	3	2	610, 700
WB I.V. into WB × C57BL	2	2	2	310*
WC × C57BL I.P. into WC × C57BL	19	17	16	180*
Juvenile hosts, WW^v (9/14)				
C57BL I.P. into 11-20 day WB × C57BL	11	9	7	780, 800, 700, 310*(4)
WC × C57BL I.P. into 6 day WC × C57BL	3	2	2	300, 475
Juvenile hosts, *WW* (4/15)				
WB I.P. into 11-13 day WB	6	2	2	75, 310
WC " " 7-13 " WC	6	2	2	45, 220
WK " " 11-12 " WK	3	0	0	

* Still alive at compilation of data, or used for other experiments after indicated interval.

the host except for *W*-series genes, may be recognized by their characteristic cell size as indicated below.

Materials and methods. Three series of experiments, differing in *W*-series genotype or age of hosts, were performed. In the first series, the anemic hosts were 24 adult (4-6 month) WW^v mice from 3 different F_1 hybrid crosses. In the second, the hosts were 14 juvenile (6-20 day) WW^v mice from 2 F_1 hybrid crosses, with their normal littermates, and in the third, the hosts were 15 juvenile (7-13 day) *WW* mice from 3 different inbred strains, with their normal littermates. In all 3 series, the implanted hematopoietic cells were obtained from livers of 15½ day old (normal *ww*) embryos, either completely isologous (except for *W*-genes) with the host, or from one of the parent-strains. The implantation technic involved injection of saline suspension of 8 x 10^6 nucleated cells, given intravenously in early experiments, and intraperitoneally in later experiments. The mice were of the following types: C57BL/6Jax, C57BL/6$W^v w$, (produced by repeated backcrossing of $W^v w$ to C57BL/6Jax), and 3 inbred strains, WB, WC, and WK, maintained heterozygous for *W* (11). WW^v mice obtained from mating of $W^v w$ parent isozygous with C57BL/6 and a *Ww* parent from WB, WC, or WK, were used as hosts in first and second series. They invariably have a severe macrocytic anemia at all ages (erythrocyte counts of young adults, 6-7 x 10^6 rbc/mm^3 in contrast to 11-12 x 10^6 rbc/mm^3 for littermate controls; mean erythrocyte volumes, 50-60 μ^3 in contrast to 39-45 μ^3 for normal littermates). In inbred strains in third experimental series, live newborn *WW* animals (erythrocyte counts approximately 1.5 x 10^6 rbc/mm^3 in contrast to 5 x 10^6 rbc/mm^3 in normal littermates) appear in almost the expected percentage (12). Nearly all die within 2 weeks of birth, but a very few, especially in the WC strain, survive to adulthood. These remain extremely anemic having erythrocyte count at 2-4 months of 4-5 x 10^6 rbc/mm^3 and M.C.V. of 70-85 μ^3. Erythrocyte counts and hematocrit determinations were made on all adult recipients before treatment, and at regular intervals after injection. In second and third series, determinations of erythrocyte levels were omitted before treatment to avoid trauma to the delicate juvenile hosts, but were started 45 days post-treatment, when all survivors were nearly full-grown mice, and were continued at regular intervals.

Results. Of 24 adult WW^v hosts implanted with fetal hematopoietic liver cells, 22 survived more than 30 days after injection (Table I), and 20 of these, from all 3 experiments, gave hematological evidence of permanent cell implantation. Two of the successfully implanted animals (WK x C57BL) survived more than 600 days after treatment, maintaining normal blood pictures to the end of their lives. Additional information on lifespan was not available since 2 of the groups (WB x C57BL, WC x C57BL) were terminated after 310 and 180 days, to use the material for another purpose. However, blood-pictures were followed in detail for 180 days in the largest series, *ww* (WC x C57BL) into WW^v (WC x C57BL). Two of the 19 adult

TABLE II. Erythrocyte Levels and Mean Cell Volumes of 16 Adult WW^v Hosts Successfully Implanted with Isologous *ww* Cells.

Days post treatment	R.B.C./mm³ 10^6 Mean	Range	Mean cell vol Mean	Range
0	6.44	5.48- 7.72	61.0	52-66
30	7.02	5.96- 7.96	58.8	52-69
60	9.41	8.16-10.70	47.9	43-54
90	9.33	6.92-10.58	45.8	42-53
120	9.31	8.30-10.54	46.2	37-53
180	9.69	8.58-11.08	44.3	34-48

hosts, with low pretreatment erythrocyte levels (5.24 and 4.79 x 10^6 rbc/mm³) died less than 60 days after treatment, and a third, also with low pretreatment level (5.24 x 10^6 rbc/mm³) survived through 180 days without evidence of implantation, and with a continuing drop in total erythrocyte count. The other 16 hosts, with higher pretreatment levels (Table II), all showed successful implantation of *ww* hematopoietic cells. There was little change in blood-picture 30 days after treatment but, between 30 and 60 days, erythrocyte numbers increased markedly and mean cell volumes decreased correspondingly (Table II), approaching values characterizing normal *ww* adults. Mean values at 90, 120, and 180 days after treatment were very similar to those at 60 days, with considerable variation between successive counts on the same animal.

TABLE III. Blood-Picture of WW^v and Littermate *ww* Mice after Injection of Isologous Embryo Blood-Forming Tissue into Juvenile Hosts.

Days post treatment	Mean, R.B.C./mm³ 10^6 WW^v (9)	*ww* (7)	Mean cell vol WW^v (9)	*ww* (7)
45	8.41	11.85	42.3	38.9
75	10.68	11.59	39.9	41.0
105	9.78	11.45	46.1	39.9
160	9.86	10.34	44.7	45.4
260	10.42	10.51	44.4	44.6

Juvenile WW^v hosts. Although no erythrocyte determinations were made before treatment of young WW^v animals, erythrocyte counts of approximately 3 x 10^6 rbc/mm³, and mean cell volumes of approximately 80-100 μ^3, were assumed at time of treatment on the basis of previous observation(12), in contrast to values of approximately 6 x 10^6 rbc/mm³ and 60-70 μ^3 for their normal littermates(12). Three of the 14 hosts died within 30 days after treatment, but 9 of remaining survivors became and remained essentially normal in blood-pictures (Table III). Erythrocyte levels for individual implanted anemics fluctuated for a considerable time, but after 75 and more days were always well above those of untreated anemic WW^v adults; mean cell volumes were, with only 2 exceptions (105 days), within the range of normal *ww* adults. The near-normal blood-cell levels of implanted anemics were maintained throughout their lives; 3 of 5 survived more than 700 days.

Juvenile WW hosts. Isologous intraperitoneal injections into 7-13 day old *WW* anemics were also successful in some cases, although the proportion of survivors was lower (4/15, Table I). It was assumed that erythrocyte counts at time of treatment were close to 2 x 10^6 rbc/mm³, with mean cell volumes of 80-100 μ^3(10). Each of the 4 *WW* individuals which survived following injections of isologous blood-forming tissue developed a near-normal blood-picture, recognized in 3 cases 45 days after injection (Table IV), in the fourth not until 105 days after injection. Two of the implanted *WW* anemics had especially short lifespans (less than 105 days after implantation); the other 2 lived less than one year,

TABLE IV. Effect of Isologous *ww* Hematopoietic Cell Implant on Blood-Picture of *WW* Mice Inj. at 7-13 Days of Age. (Erythrocyte count untreated surviving adult *WW*, 4-5 × 10^6 R.B.C./mm³; M.C.V., 70-85 μ^3.)

	WW from WB strain				*WW* from WC strain			
	#1		#2		#3		#4	
Days post implant	R.B.C./mm³ 10^6	M.C.V., μ^3	R.B.C./mm³ 10^6	M.C.V., μ^3	R.B.C./mm³ 10^6	M.C.V., μ^3	R.B.C./mm³ 10^6	M.C.V., μ^3
45	4.83	62	11.78	41	9.77	40	8.41	46
75			10.69	42				
105	10.06	40	Died with in-		11.99	36	Died	
210	8.24	38	fection		9.44	42		
310	7.40	45			Died			

maintaining normal *ww* cell volumes, and erythrocyte numbers slightly below normal *ww* levels.

Discussion. All evidence presently available on genetically anemic (WW^v, WW) mice supports the hypothesis that isologous normal donor cells implant and continue to function autonomously according to their *ww* genotype. The present data clearly demonstrate that radiation to prepare the marrow "bed" is not essential for implantation of isologous normal embryonic blood-forming tissue, success having been obtained with WW^v adult and with WW^v and WW juvenile hosts without previous radiation treatment. The highly cellular marrow of WW^v adults(13) did not prevent implantation of isologous cells, the proportion of success (20/24) being similar to that found (16/18) following 200 r whole-body radiation (9). In adult animals injected without prior radiation, a considerable period of time, 30 to 60 days in contrast to 18 days with 200 r radiated hosts, elapsed before the effect of implanted blood-forming cells was apparent on the blood-picture of host. Failure of implantation in adult WW^v individuals with especially low pretreatment levels may mean that health of host plays some part in fostering growth of the implant. After a rise in erythrocyte count had been observed, large fluctuations in successive counts, extending over prolonged periods of time, were frequently observed, but always culminated in a permanent high level—only slightly lower than that for control *ww* adults or adult WW^v mice injected after exposure to 200 r.

Similar fluctuations and delay in establishment of a near-normal blood-picture were observed with implantation of juvenile WW^v hosts. Using juvenile hosts, 2 obvious factors, related to health of host, may contribute to mortality after injection. Some animals may die before the implanted cells can establish themselves, since only 25-31% of WW^v (and less than 10% of WW individuals) spontaneously survive the critical pre-weaning period of active growth(11). Moreover, the trauma of intraperitoneal injection may affect longevity of these poorly viable hosts. The difference in proportion of success with WW^v juvenile hosts (9/14) and with WW hosts (4/15) fits both these suggestions. The short lives of successfully implanted WW hosts suggest either that undesirable side-effects are produced by the operation as presently practiced, or that the lifespan of WW individuals may depend upon genic effects other than those determined by the implanted blood-forming tissue.

Many of these observations fit well with the hypothesis of competition between a large body of indigenous defective, slow-acting blood-forming tissue of the genetically anemic host(14) and an initially small implant of rapidly functioning blood-forming tissue from the genetically normal donor. This phenomenon of increasing effect from the implant may be different from that observed if both host and donor tissue had normal blood-forming capacity. If so, the difference would almost certainly favor permanent implantation after normal-into-anemic injections more than after normal-into-normal injections.

The demonstration that isologous implantation can take place without X-ray preparation of host solves some, but by no means all, of the problems of therapy of blood-forming defect of genetically anemic *W*-series mice.

It is a step forward to know that the marrow "bed" of the radiosensitive hosts need not be subjected to radiation, especially since no level of radiation has been observed which allows survival with homologous implantation (11). The small proportion of surviving WW^v and WW juvenile hosts suggests, however, that attempts should be made to minimize trauma to the host, and to develop methods assuring rapid implantation and proliferation of the injected cells.

Since it seems possible that extreme radiosensitivity might be encountered in cases of genetically-conditioned human hypoplastic anemias, the demonstration of implantation without radiation in genetically anemic mice is of interest. However, for this finding to have any carry-over into therapy of human genetic defects of hematopoiesis, it is necessary to find methods which will promote implantation of homologous blood-forming tissue, since with therapy of human genetic defects, it is nearly impossible to obtain isologous cells for implantation. Experiments

testing possibilities for implanting homologous normal cells in anemic hosts are in progress.

In spite of these limitations the successful cases of permanent isologous implantation of normal blood-forming tissues in genetically anemic mice, including the lethal *WW* type, without prior radiation treatment, suggest that if methods for circumventing the homograft reaction can be found, it may be possible to use cell implantation generally for the therapy of genetic defects in erythropoiesis.

Summary. Successful implantation, without x-irradiation or other pretreatment of host, of isologous blood forming tissue from normal (*ww*) mouse fetal liver in viable severely anemic recipients (20 of 24 adult WW^v mice, 9 of 14 juvenile WW^v mice) and in lethally anemic recipients (4 of 15 juvenile *WW* mice) has been demonstrated. Criteria of success were permanent increase in rbc/mm^3 to near-normal levels, and decrease of mean cell volume from macrocytic to normocytic levels. These changes appeared in WW^v adult anemic hosts longer after cell injection than in similar transplant experiments with WW^v hosts subjected to 200-r whole-body irradiation. These findings fit well with the hypothesis of competition between slow-acting indigenous blood forming tissue of genetically anemic hosts and initially small implants of rapidly functioning blood forming tissue from genetically normal donors. If methods for circumventing homografts can be found, cell implantation may be generally useful for therapy of genetic defects in erythropoiesis.

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Effect of Degree of Encapsulation upon Virulence of *Cryptococcus Neoformans.** (25090)

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Capsule formation has been associated with virulence of many microbial species such as *Diplococcus pneumoniae, Bacillus anthracis, Hemophilus influenzae* and *Klebsiella pneumoniae.* However, the chemical nature of capsular substances formed by different microbial species is dissimilar, varying from complex polysaccharides composed of units of glucose, fructose, galactose, mannose, sugar acids and amino sugars, to polypeptides such as polymers of glutamic acid. The capsule of *Cryptococcus neoformans* contains 2 complex polysaccharides; an amylose of the starch group, and a serologically active pentosan which upon hydrolysis, yields units of xylose, mannose, galactose and glucuronic acid(1). To date, no single chemical constituent of cap-

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sular substance has been directly associated with virulence. Although literature contains many references re. encapsulation on virulence of bacterial species, studies of encapsulated yeasts are limited. Drouhet and Segretain(2) noted that an encapsulated, mucoid, dissociant strain of *C. neoformans,* isolated from a patient, was more virulent to mice than the thinly encapsulated, parent yeast strain. In contrast, Kao and Schwarz(3) failed to observe correlation between capsule thickness of *C. neoformans* and virulence in white Swiss mice with strains recovered from pigeon nests. Gadebusch(4) observed that variant strains of *C. neoformans* which possessed large capsules resisted phagocytosis by mouse polymorphonuclear leucocytes while thinly encapsulated yeast variants were readily ingested. The preceding studies were performed, however, before precise nutritional requirements of *C. neoformans* had been defined by Littman (1). In these studies the organism possessed an absolute requirement for thiamine and its moieties, a specific need for glutamic acid family of amino acids, and need for certain carbohydrates. A synthetic basal substrate, *Cryptococcus* Capsule Medium‡ (CCA), which satisfied complete nutritional need of *C. neoformans* and caused it to synthesize abundant capsular substance, has been described (1). It has also been observed that capsule synthesis diminishes rapidly when yeast is transferred from the medium to a dextrose-peptone agar such as Sabouraud dextrose agar§ (SAB). This provides a means for determining comparative virulence of the same yeast strain existing in different degrees of encapsulation.

Materials and methods. C. neoformans (D strain), isolated at our hospital from a fatal case of cryptococcal meningitis, was subcultured on both CCA and SAB agar media 4 days at 37°C. Culture growth in each medium was suspended in sterile, physiological saline. Measurements of yeast cell diameter, including the capsule, were made with ocular micrometer, using diluted fungicidal India ink mount.‖ Photometric measurements of saline suspensions of the organisms at 650 mμ revealed light transmission values of 19.3 and 19.5%, representing plate counts of 17.6 X 10^6 and 4 X 10^6 cells/ml, respectively. Ten-fold, 1000-fold, and 10,000-fold saline dilutions of each cell suspension were prepared for injection. Ninety 6-week-old, female, white Swiss mice, each weighing approximately 15 g, were divided into 6 groups of 15 mice each. Three groups received intracerebral injections of 0.0625 ml of each of the 3 dilutions of thickly encapsulated CCA yeast cells, while the 3 remaining groups received thinly encapsulated SAB cells. Intracerebral injections were made midway between eye and ear, using 0.25 ml hypodermic syringe and 26 gauge 3 mm needle. Mice were weighed and examined each day for hydrocephalus. Upon death, gross pathological examination was made of all organs including brain. At autopsy, a platinum loop was inserted into the cerebral matter at site of injection and an India ink mount was prepared. Capsules of 30 yeast cells in each brain preparation were measured with ocular micrometer. Mouse survival time data were computed according to a modification of the nomograph method of Litchfield (5,6). Data were recorded as per cent cumulative fatality and plotted on 2-cycle logarithmic probability paper, with time expressed in days on logarithmic scale. Using least squares method, the best straight line was constructed. Standard deviation was computed by substitution of effective fatality times (ET_{80}, ET_{50} and ET_{20}) in the equation: Stand. dev. (S): $\frac{ET_{80}/ET_{50} - ET_{50}/ET_{20}}{2}$.

Results. Marked differences were observed in colonial and microscopic appearance of test strain of *C. neoformans* on SAB and CCA agar media (Fig. 1). Growth of the organism on

‡ Containing/liter of medium: KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 2g; $MgCl_2.6H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.02; $FeCl_2.4H_2O$, 0.04 g; $MnCl_2.4H_2O$, 0.0015 g; $Na_2MoO_4.2H_2O$, 0.0015 g; thiamine, 0.001 g; maltose, 5 g; sucrose, 5 g; sodium glutamate, 2 g; washed agar, 20 g; double distilled water, 1000 ml; pH 7.0.

§ Containing/liter of medium: neopeptone (Difco), 10 g; dextrose 40 g; agar 15 g; pH 5.6.

‖ India ink, 15 ml; solution of Merthiolate (Lilly) 1:1000, 30 ml; Tween-80 1:100 aq. solution, 0.1 ml; filtered before use.

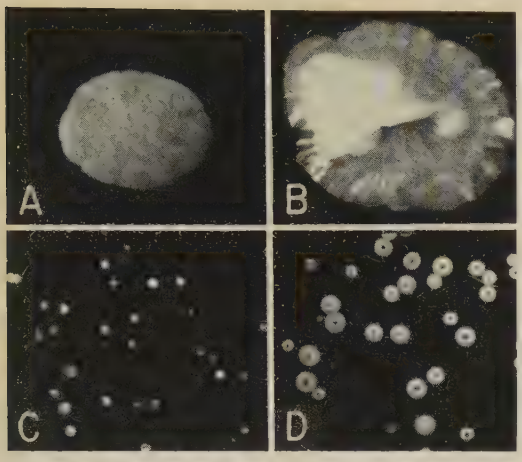

FIG. 1. Effect of culture substrate on *in vitro* encapsulation of *Cryptococcus neoformans*. (A) Giant colony on Sabouraud dextrose agar (SAB), 16 days at 37°C, ×.78. (B) Giant colony on *Cryptococcus* capsule agar (CCA), 16 days at 37°C, ×.78. (C) India ink mount of growth from SAB colony showing absent or sparse capsules, phase microscopy, unstained, × 117. (D) India ink mount of growth from CCA colony showing abundant capsules, × 117.

CCA medium was raised, mucoid, sectored, viscous, profuse and flowing; as a consequence a large colony was formed (Fig. 1B). Microscopic examination of this colony with India ink mount showed thickly encapsulated yeast cells (Fig. 1D). The colony on SAB medium, however, was considerably smaller (Fig. 1A). It was butyrous in consistency, showed sectoring, and appeared flat, dry and dull. Microscopic examination with India ink mount revealed, for the most part, thinly encapsulated cells (Fig. 1C). Of 100 cells grown on SAB, the average cell diameter, including capsule, was 9.12 μ, while CCA cells averaged 16.04 μ. While only 1% of the SAB cells were encapsulated, as many as 92% of the CCA cells demonstrated capsules. The majority of CCA cells possessed capsules that measured more than 14.4 μ in diameter.

Virulence of both CCA and SAB yeast cells in mice after intracerebral injection was approximately equal. This occurred over dose range of 25 to 1,100,000 cells (Fig. 2). Mouse mortality was directly proportional to intracerebral dose of yeast cells, without regard to their initial state of encapsulation. Thus, median effective fatality time (ET_{50}) for 110 CCA yeast cells was 14.8 days (Table I). Increasing the dose 10,000 times to 11 X 10^5 cells reduced ET_{50} value by only half, or 7.2 days. ET_{50} values for each dose of yeast cells were statistically similar for both types (Table I) and the curves of ET_{75}, ET_{50} and ET_{25} values were parallel.

Both thinly encapsulated SAB cells and thickly encapsulated CCA cells produced equally thick capsules after intracerebral injection. No appreciable differences in incidence of hydrocephalus were observed in mice that had received either type of yeast cell. Mice receiving the heaviest intracerebral dose of yeast cells died in 5 to 11 days (Fig. 2, Table II). Group II mice, given a smaller dose of yeast cells, died in 8 to 22 days, while Group III mice which had received the

TABLE I. Comparison of Median Effective Fatality Time (ET_{50}) in White Swiss Mice Produced by Test Strain of *Cryptococcus neoformans* Grown on *Cryptococcus* Capsule Agar (CCA) and Sabouraud Dextrose Agar (SAB).

Group	Intracer. dose, yeast cells	Culture medium	ET_{50} (days)	Stand. dev. (days)
I	11 × 10^5	CCA	7.2	1.22
	2.5 × 10^5	SAB	8.2	1.20
II	11 × 10^3	CCA	11.0	1.32
	2.5 × 10^3	SAB	13.2	1.33
III	110	CCA	14.8	1.39
	25	SAB	18.0	1.55

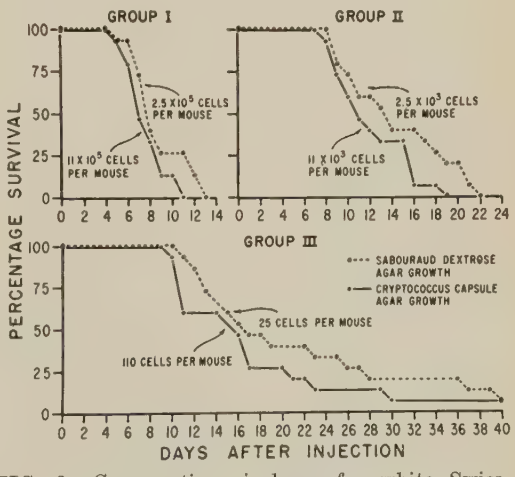

FIG. 2. Comparative virulence for white Swiss mice of thinly encapsulated SAB cells and thickly encapsulated CCA cells of *Cryptococcus neoformans*, following intracer. inj. of 0.0625 ml saline suspensions, 15 mice/group (arithmetic plot).

TABLE II. Comparison of Diameters of *Cryptococcus neoformans* Cells, Including Capsules, Grown on *Cryptococcus* Capsule Agar (CCA) and Sabouraud Dextrose Agar (SAB) (*In Vitro*) and following Intracerebral Injection (*In Vivo*).

Group	Intracer. dose of yeast cells	Culture medium	Avg capsule diameter (μ)* *In vitro*	Avg capsule diameter (μ)* *In vivo* mouse brain	% of mice with hydrocephalus on death	No. of days required to cause death
I	11×10^5	CCA	16.04	13.3	86	5-11
	2.5×10^5	SAB	9.12	14.1	80	5-14
II	11×10^3	CCA	16.04	13.1	93	8-19
	2.5×10^3	SAB	9.12	14.5	100	9-22
III	110	CCA	16.04	13.5	"	10-30
	25	SAB	9.12	14.3	"	11-40

* Avg of 100 cells.

smallest inocula, succumbed in 10 to 40 days. Mice that succumbed first (Group I) showed 80 to 86% hydrocephalus, while animals that survived for longer periods all developed hydrocephalus.

Discussion. It is known that strains of *C. neoformans,* cultivated on dextrose-peptone agar, vary considerably not only in thickness of their capsules but also in virulence for mice(7). Littman and Schneierson(7) noted that of 144 mice injected intracerebrally in pairs with 3 X 10^6 yeast cells, derived from 72 "pigeon" strains, 18% succumbed 10 days after injection, 40% within 3 weeks and 100% within 73 days. Thus it would seem that mouse virulence test for identification of *C. neoformans* is of limited diagnostic value where speed of diagnosis is desired. Cultural and biochemical procedures are preferred for identification of the organism(7).

In assaying the virulence of different strains that possess thin or thick capsules, the inherent virulence of the organism cannot be attributed solely to capsule size, since factors of strain virulence other than encapsulation may be involved. To eliminate the factor of strain differences, virulence studies herein reported were limited to a single strain of the organism. *In vitro* cultivation on CCA and SAB agar media permitted use of cells of the same strain since they differed widely in degree of encapsulation.

Assay of virulence of thinly and thickly encapsulated cells, derived from the same strain of *C. neoformans,* has not been previously reported. Our results show they possess the same degree of virulence for white Swiss mice. Thinly encapsulated SAB yeast cells also produced equally thick capsules *in vivo* as did thickly encapsulated CCA cells. The prompt increase in *in vivo* encapsulation of SAB cells is due probably to the fact that thiamine, glutamic acid, and certain carbohydrates and minerals, essential for growth of *C. neoformans,* exist in optimal concentrations in brain and CSF(1).

In computing mouse survival time according to the method of Litchfield(5,6), CCA and SAB yeast cells produced similar cumulative fatality curves and exhibited similar ET_{50} values. The results also show that, within certain limits, mouse virulence test for *C. neoformans* is dependent upon the intracerebral dose of yeast cells. For example, ET_{50} value of *C. neoformans* for white Swiss mice decreased from 18 to 8.2 days, with increased dosage of SAB yeast cells (Table I).

Summary. Thinly encapsulated *Cryptococcus neoformans* cells were equally as virulent for white Swiss mice as thickly encapsulated cells of the same strain. Yeast cells initially possessing thin capsules promptly developed thick ones upon intracerebral injection into mice, and upon subculture to synthetic capsule medium. Both types of cells produced hydrocephalus to an equal degree. Degree of encapsulation is not a factor in virulence of *Cryptococcus neoformans* for white Swiss mice.

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Electron Microscopic Study of Human Cholestasis.* (25091)

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Pathogenesis of jaundice associated with intrahepatic cholestasis is not established despite extensive research for many years. Jaundice with clinical and laboratory findings suggestive of biliary obstruction and without indications of injury of liver cells or hemolysis is present in the absence of a demonstrable obstacle in intrahepatic or extrahepatic bile ducts. On the basis of clinical, functional and light microscopic investigations, the alteration was localized into different sites, such as liver cells(1,2), Kupffer cells(3), cholangioles or ductules(4) as well as into lining of bile canaliculi(5,6). To throw light on this phenomenon, the ultrastructure of liver in human extrahepatic biliary obstruction, intrahepatic cholestasis and other types of hepatic jaundice were studied under the electron microscope. For comparison, liver of rats with experimental injuries from ethionine(7) and experimental extrahepatic biliary obstruction were studied. Previous electron microscopic observations have indicated abnormal communication between dilated bile canaliculae and perisinusoidal tissue spaces(8).

Material and methods. Biopsy specimens of liver of patients with hepatic injury and jaundice following intake of chlorpromazine, chlorpropamide, norethandrolone, and with primary biliary cirrhosis served as examples of intrahepatic cholestasis. Extrahepatic biliary obstruction was represented by carcinoma of the pancreas, jaundice associated with liver cell injury by viral hepatitis, and one case of chronic idiopathic jaundice was included. Small pieces of these as well as of livers of experimental animals mentioned above were fixed in 2% buffered ice-cold osmium tetroxide and thin sections examined with Philips model EM 100 B electron microscope. Other parts of tissue were routinely fixed in formalin and examined by standard light microscopic technics, which included particularly the periodic acid Schiff reaction after glycogen removal with diastase for demonstration of carbohydrate-bound protein.

Results. The bile canaliculus of normal human beings and rats appeared under the electron microscope as a round or elliptical lumen approximately one μ in diameter into which fine fingerlike projections extended (microvilli) (Fig. 1). It was separated from nearby extensions of the perisinusoidal tissue spaces by closely approximated straight cell borders. Liver cells projected as less regular microvilli into tissue spaces including their intercellular narrow extensions referred to above. Under the light microscope tissue spaces and some of the extensions gave a faint PAS reaction. Each liver cell usually had more than one bile canaliculus on its borders. The inner border of ductules was also lined by microvilli which were shorter and further apart than in liver cells.

In cases of intrahepatic biliary obstruction studied, the bile canaliculi appeared less numerous (Fig. 2). A few were the same size as normal canaliculi whereas others were dilated to a maximal diameter of 4 μ; in the latter, electron opaque debris was noted. In all canaliculi the microvilli were either entirely absent or further apart than normal and shortened, stunted or irregularly shaped. In a few instances a communication between dilated microvilli and extension of tissue spaces was

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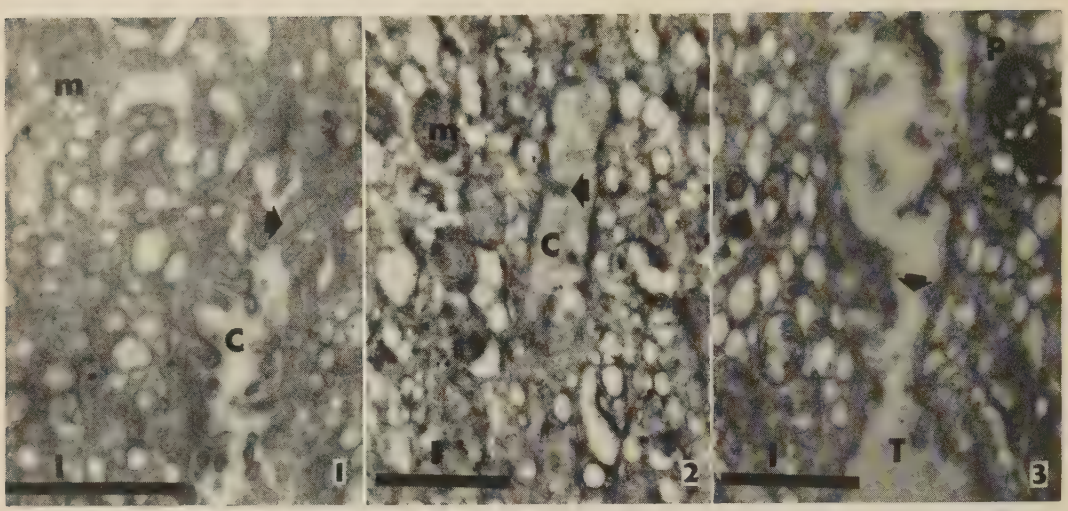

FIG. 1. Normal human bile canaliculus (c) between 2 liver cells showing long delicate microvilli (arrow). A mitochondrion (m) is in left upper corner. The round cystic structures are part of endoplasmic reticulum. Heavy line in left lower corner is one micron. (× 21,334).

FIG. 2. Bile canaliculus (c) between 2 liver cells in a patient with cholestasis following administration of chlorpropamide showing few and abnormal microvilli (arrow). Heavy line in left lower corner is one micron. (× 15,334).

FIG. 3. Bile canaliculus (c) in a patient with extrahepatic biliary obstruction due to carcinoma of the pancreas showing communication (arrow) with perisinusoidal space (T) and virtual absence of microvilli. Dark body (P) in the right upper corner is lipofuscin. Heavy line in left lower corner is one micron. (× 16,000).

noted by separation of borders of neighboring liver cells. The only alteration of the cytoplasmic detail of the liver cell was in the endoplasmic reticulum. Under the light microscope the PAS reaction in the perisinusoidal area was increased and in the liver cells PAS positive granules were found in increased numbers, some of them bile-pigmented. Moreover, bile canaliculi contained PAS positive material in addition to bile. Bile ductules were not recognized in specimens studied so far. In extrahepatic biliary obstruction, similar changes were found except that fewer bile canaliculi were noted particularly in rats 3 weeks after ligation of the bile duct. Some bile canaliculi were conspicuously dilated up to 10 μ in diameter, contained much radiopaque debris, apparently bile plugs. Many canaliculi were formed by several surrounding liver cells. Also, communications with tissue spaces were readily apparent (Fig. 3). The microvilli in all bile canaliculi were absent or considerably distorted. The cytoplasmic and the light microscopic changes were similar to those in intrahepatic cholestasis. In viral hepatitis or in rats with ethionine intoxication despite conspicuous alteration of cytoplasmic detail including mitochondria and endoplasmic reticulum, the bile canaliculi and microvilli appeared unaltered as a rule. Only in some instances of viral hepatitis slight dilatation with alteration of microvilli and radiopaque intraluminal precipitates were noted. Also in chronic idiopathic jaundice with heavy cytoplasmic pigment deposition, normal bile canaliculi and microvilli were noted.

Discussion. The electron microscopic examination indicated a characteristic alteration of the canalicular border of the liver cell with abolition or distortion of the normally present microvilli. The latter can be considered as a structural expression of interphase or membrane processes on the biliary border of the liver cell. In extrahepatic biliary obstruction, these alterations are presumably the result of increased hydrostatic pressure which is also reflected in dilatation of the canaliculi and their subsequent communication with the widened tissue spaces. This regurgitation previously described in experimental animals(8) may account for at least some of the hyperbilirubinemia. Moreover, alteration of microvilli

interferes with recognition of bile canaliculi which appeared reduced in number. The precipitation of bile and presence of protein-bound carbohydrates in the biliary passage suggests an alteration of the bile which may produce an intrahepatic component of the cholestasis in extrahepatic biliary obstruction.

In intrahepatic cholestasis exemplified by a variety of drug-induced alterations(9), the same disturbance of microvilli exists. This is presumably primary, although a hydromechanical factor is reflected in the dilatation of the canaliculi. However, the latter is far less conspicuous than in extrahepatic obstruction and possibly is the result of the inspissation of bile. In both intrahepatic cholestasis and extrahepatic obstruction, the fine structure of liver cell cytoplasm does not suggest a significant functional involvement of the interior of the cell. The basic similarities despite quantitative differences in the picture of extrahepatic biliary obstruction and intrahepatic cholestasis explains why the morphologic picture does not assist in differential diagnosis of both conditions. In other types of hepatic jaundice such as in chronic idiopathic jaundice and viral hepatitis, the bile canaliculi are not necessarily changed. The focal alterations in viral hepatitis might reflect the known intrahepatic cholestatic component occurring in this condition which sometimes is in the foreground of the clinical picture. Involvement of the ductules or cholangioles in both types of cholestasis has not been demonstrated and therefore at this time neither support for nor opposition to the concept of involvement of these structures reflected in the conventional name "cholangiolitis" can be offered.

Summary. In both extrahepatic biliary obstruction and intrahepatic cholestasis, alteration of the bile canalicular wall of liver cells and distortion of normally present microvilli can be demonstrated by electron microscopy. The functional defect is localized into membrane of liver cells, the more so since cytoplasm of liver cells does not necessarily show significant alterations. It is assumed that the lesion in extrahepatic biliary obstruction results from increased hydrostatic pressure while it cannot be decided whether in intrahepatic cholestasis it is primary or also secondary to increased pressure. Microvillous changes are not found in chronic idiopathic jaundice and only focally in viral hepatitis with liver cell injury possibly as the result of intrahepatic cholestatic component. In both types of cholestasis, communication between dilated bile canaliculi and perisinusoidal tissue spaces may account by regurgitation for at least part of the jaundice.

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Observations on Glyoxylic Acid Reaction in Mental Disease.* (25092)

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Riegelhaupt(1,2) reported that performance of a modification of the Hopkins-Cole qualitative test for urinary tryptophane(3) yields a positive result in 76% of schizophrenic patients studied. It has been suggested that this reaction might be related to unusual indolic compounds described in schizophrenic urine(2,4). To assess and extend these findings, the diagnostic validity and specificity of the reaction were tested in a controlled series of subjects.

Method. Two ml of fresh, filtered, early morning urine specimen were shaken with 0.5 ml of butanol-acetic acid solvent (n-butanol 60: glacial acetic acid 15: distilled water 25, made fresh daily), allowed to stand and shaken again when separation had occurred. After adding 1 ml of 2% glyoxylic acid, 1 ml of concentrated sulfuric acid was layered slowly into the tube in ice bath. Samples were read at 15 minutes for presence of red-violet color at acid-urine interface which constituted a positive test. Development of predominately brown, yellow, or colorless rings was called a negative test. All intermediate colors were arbitrarily and subjectively assigned to one of the 2 groups. Subjects were selected at random from hospital population and, following a brief trial period, 113 separate tests were made as "unknowns" with respect to contributing subjects status as schizophrenic, non-schizophrenic patient, or normal (employee or staff member). Patients diagnosed as schizophrenic by staff psychiatrists at time of current admission study comprised the schizophrenic group. The non-schizophrenic group included a large number of affective psychoses, neurotics, and character disorders. Post-experimental analysis of non-experimental variables necessitated elimination of 14 patients who were receiving medication (phenothiazine derivatives or meprobamate) known to interfere with the test. This fact is responsible for the slight deficiency in number of schizophrenics relative to the other 2 study groups in Table I. The groups are comparable with respect to age except within the schizophrenic category where females are considerably older and males considerably younger than control groups. Dietary and drug factors were comparable for the study groups; there was no deficiency evident and specific excesses, such as coffee, were controlled when encountered. Results of the test are summarized in Table II. A positive test was associated with clinical diagnosis of schizophrenia in 62.5% of 24 cases studied, whereas only 8% of 75 non-schizophrenic subjects gave a positive reaction. Positive tests were encountered equally among normals and non-schizophrenic patients. Individuals giving positive tests were distributed indifferently within the schizophrenic group with respect to age and sex.

Discussion. Our results are roughly equivalent to those reported by other workers(2,4); a somewhat lower percentage of both schizophrenics and non-schizophrenics gave positive tests which may be related to the concentration of glyoxylic acid used, or simply to subjective differences in the observers. With decreased sensitivity, an increase in discrimination and validity is achieved.

TABLE I.

Diagnostic category	No.	Avg age	♂	Age	♀	Age
Schizophrenic	24	40.0	10	26.5	14	49.1
Non-schizophrenic	75	38.3	24	40.5	51	38.2
Patients	39	39.6	9	38.3	30	40.7
Normals	36	36.8	15	41.1	21	35.6

TABLE II.

Diagnostic category	Positive test	Negative test	Total No.
Schizophrenic	15	9	24
Non-schizophrenic	6	69	75
Patients	3	36	39
Normals	3	33	36

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To what degree does the test permit us to distinguish between the 2 groups, one schizophrenic and the other non-schizophrenic? Chi-square analysis yields a value well in excess of that corresponding to P value of .001 defining the great unlikelihood that this distribution could have occurred by chance. The distinction between schizophrenics and either non-schizophrenic patients or normals is equally non-fortuitous. All of these differences are more striking than can be accounted for by age and sex differences alone though these factors have not been completely controlled in our study.

As a diagnostic test the reaction has obvious limitations; it does not occur in all schizophrenics, its specificity is open to question, and its reproducibility has not been studied.

Conclusions. A positive result with glyoxylic acid test was obtained in 62.5% of schizophrenic patients, while only 8% of non-schizophrenics gave a positive test. Though non-specific the test affords a statistically significant distinction which cannot be explained by the non-experimental variables. Because of the chance that the test has a significant association with the disease, schizophrenia, further investigation seems warranted.

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Maturation of Succinic Dehydrogenase and Cytochrome Oxidase in Neonatal Rat Kidney. (25093)

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It is well recognized that functional differences exist between the kidneys of immature and adult animals(1). Although differences are apparent in organization and structure of such kidneys(2) integration of this data has not been accomplished. A depletion of histochemically demonstrable succinic dehydrogenase and cytochrome oxidase and depressed oxygen consumption have been recently demonstrated in the kidneys of nephrotic rats by Fisher and Gruhn(3,4). These changes could be correlated with the degree of proteinuria exhibited by these animals although it was not evident whether this enzymatic alteration was a causative factor or a sequel of proteinuria. Since several references(5,6,7) to occurrence of proteinuria in newborns have been made it appeared worthwhile to explore the relationship of proteinuria and these respiratory enzymes in kidneys of maturing rats. Information derived from such a study also appeared pertinent in providing cytochemical evidence concerning differences in renal function of neonates and adults since oxidative enzymes are considered essential for proper function of the tubular portion of the nephron. Developmental changes in renal alkaline phosphatase have been noted previously(8).

Material and methods. Four to six littermates of the Wistar strain were sacrificed at 1, 7, 12, 15, 18, 24, 27, 30, 39, 63 and 72 days after birth. One kidney was immediately frozen on dry ice and the other placed in Zenker's acetic fluid. Blocks of fresh frozen material were stored at –20°C until the last animal was sacrificed and all fresh frozen material was sectioned at 6 μ in a cryostat and simultaneously stained for demonstration of succinic dehydrogenase by the method of Rosa and Velardo(9) and cytochrome oxidase (G-nadi oxidase)(10). Previous experience indicated that storage of fresh frozen tissue for similar periods did not alter the appearance of these enzymes. Sections prepared from tissue fixed in Zenker's acetic fluid were cut at 5 μ and stained with hematoxylin and eosin, periodic acid-Schiff and phosphotungstic acid hematoxylin technics. Each group contained

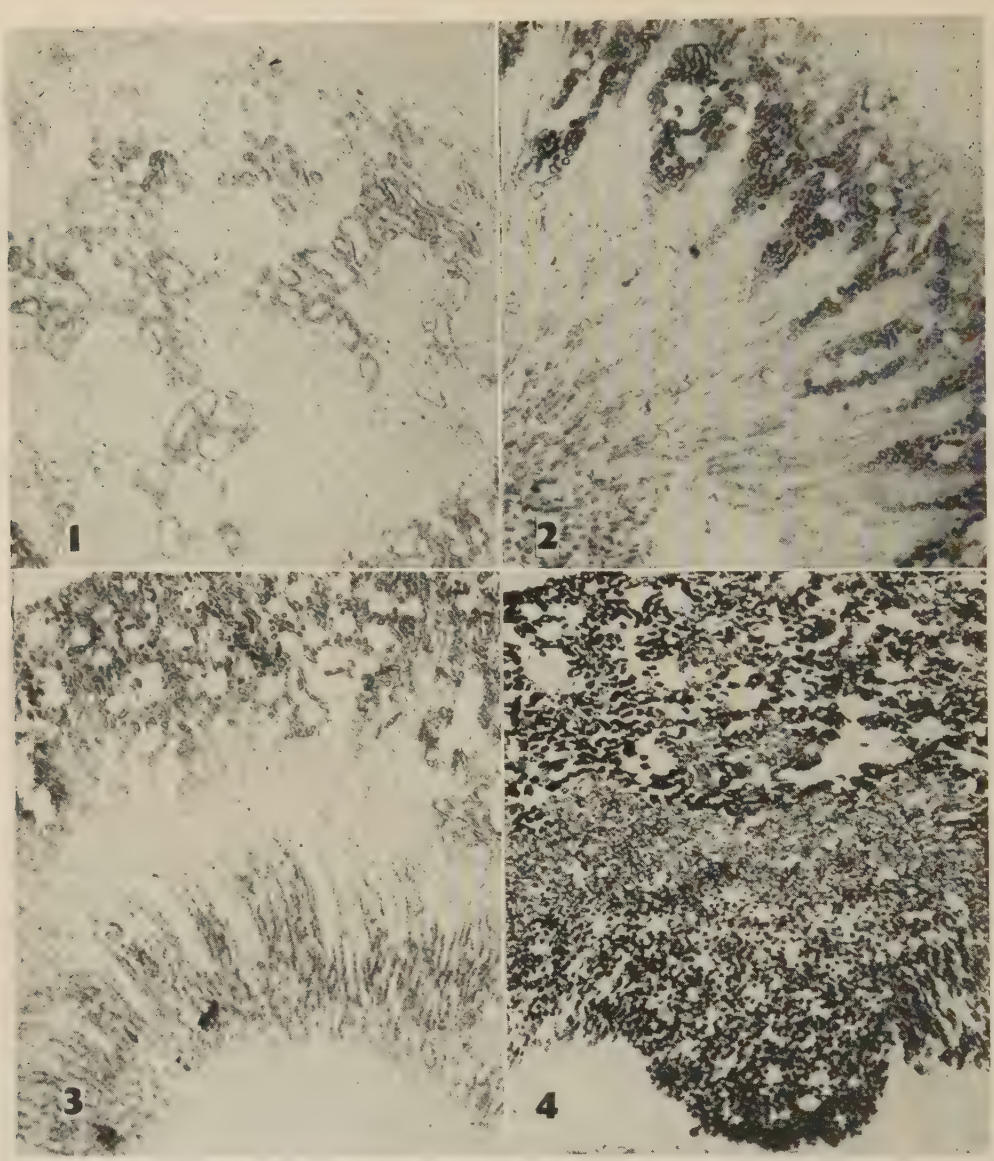

FIG. 1. Succinic dehydrogenase (appearing black) in isolated tubules of cortex of kidney of rat 1 day old. (× 65)

FIG. 2. Succinic dehydrogenase (appearing black) in outer cortical zone with slight reaction in neocortical tubules of neonatal rat 15 days old. (× 30)

FIG. 3. Cytochrome oxidase (appearing black) in cortical zones and moderate activity in outer medullary area corresponding to ascending loops of Henle in neonatal rat kidney 22 days old. (× 30)

FIG. 4. Succinic dehydrogenase (appearing black) with adult distribution and staining intensity in kidney of neonatal rat 39 days old. (× 30)

at least one female. Amount of protein excreted by newborn rats was determined by placing a suture ligature about the external genitalia of groups of 4 rats at 2, 4 and 16 days after birth. Animals were subjected to laparotomy 24 hours later and urine aspirated from their urinary bladders into a clean syringe through a #23 needle. Total protein was estimated according to method of Looney and Walsh(11). Estimation of protein reabsorption within the kidney was performed on 4 newborns 4 days after birth. Each re-

TABLE I. Distribution and Staining Intensity of Succinic Dehydrogenase and Cytochrome Oxidase in Neonatal Rat Kidney.

Age (days)	OCZ	ICZ	OMZ
1	1+ *		
7	2–3+*	±	±
12	2–3+*	"	"
15	2–3+	"	"
18	4+	1+	"
24	"	"	1+
27	"	2+	1–2+
30	"	"	3+
39	"	"	4+
63	"	"	"
72	"	"	"
Adult	"	"	"

* Neocortical tubules negative.

OCZ, outer cortical zone; ICZ, inner cortical zone; OMZ, outer medullary zone.

ceived an intraperitoneal injection of 2.5 mg of Evans blue dye and sacrificed 24 hours later.

Results. Distribution and staining intensity of both succinic dehydrogenase and cytochrome oxidase were similar. A few tubules in the outer cortical zone which includes convoluted segments of proximal convoluted and distal convoluted tubules exhibited faint enzymatic activity 24 hours after birth (Table I and Fig. 1). Tubules in the neogenic area, however, were devoid of such activity until 15 days after birth (Fig. 2). At this time and shortly thereafter tubular components of the outer cortical zone assumed an adult appearance (Fig. 3). Complete maturation of tubules in inner cortical zone corresponding to straight segments of proximal convoluted tubules and portions of the ascending limbs of Henle was not apparent until 27 days of post natal life. The last zone to acquire mature characteristics corresponded to that topographical area comprised of ascending limbs of Henle located in the outer medullary zone at 39 days (Fig. 4). The variable lack of enzymes prior to 39 days was accompanied by absence of mitochondria. Brush borders, as evidenced by periodic acid-Schiff stain, were absent in the proximal convoluted tubules of the neogenic zone but became evident with its maturation. There was no apparent difference in male or female newborns.

Protein excretion/24 hours, as estimated from bladder aspirates following ligation of external genitalia, was 7-15 mg. Our repeated control determinations revealed a normal adult value of 7-29 mg/24 hours.

Kidneys of newborns receiving Evans blue were only faintly stained macroscopically. Their urines also were only mildly colored by the dye. Microscopic examination of fresh frozen sections failed to disclose intracellular droplets. These findings are similar to those of normal adult rats receiving intravenous injections of Evans blue, but are unlike those of nephrotic animals in which renal cortex and urine are intensely stained and blue droplets may be readily discerned within epithelium of portions of proximal convoluted tubules(3).

Discussion. Failure to observe proteinuria or alteration in renal tubular reabsorption of protein in the neonatal rat precludes comment concerning relationship of this phenomenon and depletion of respiratory enzymes noted. However, it does indicate the necessity for reevaluation of references to proteinuria in newborn. It also appears significant in the light of recent electron microscopy relating the ultrastructure of neogenic glomeruli to that observed in glomeruli considered characteristic of the nephrotic syndrome(12).

Absence of histochemically demonstrable succinic dehydrogenase and cytochrome oxidase in various tubular segments of the immature kidney of the rat complements the findings of Cutting and McCance(13) who noted decreased oxygen consumption by such kidneys. Recognition of the segmental development of these respiratory enzymes provides further cytochemical evidence for differences encountered in neonatal and adult renal function and offers an opportunity for more precise physiologic and structural correlation.

Summary. Succinic dehydrogenase and cytochrome oxidase undergo maturation in respect to distribution and staining intensity in the neonatal kidney of the rat. Recognition of this event provides cytochemical evidence of functional immaturity in the neonatal kidney. Proteinuria or altered tubular reabsorption of protein was not observed in newborns of this species.

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Effect of Lipoprotein Lability on Immunological Properties of Human Low Density Lipoproteins.* (25094)

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The effects of aging, freezing and thawing, and heating on immunological properties of human low density lipoproteins were studied to estimate the extent of changes produced by these procedures. Various reports(1-6) have indicated that lipoproteins are labile molecules readily denatured by auto-oxidation and aggregation reactions which may also alter the serological properties of the lipoproteins.

Methods. S_f 3-9 and S_f 10-400 lipoprotein fractions were prepared from human serum by ultracentrifugal flotation procedures(7), characterized by chemical composition(3), and injected into rabbits to produce antisera (7). The lipoprotein antisera did not react with human albumin, γ-globulin, high density lipoproteins, or lipoprotein free serum(7). Agar diffusion and precipitin ring tests were used in the immunological studies. The agar diffusion experiments were performed by plate method of Rheins *et al.*(8). In precipitin ring tests, doubling dilutions of an S_f 3-9 lipoprotein fraction in 0.2 ml volumes were layered over 0.2 ml of anti S_f 3-9 serum in precipitin tubes. These tubes were incubated at room temperature 30 minutes, then observed for appearance of a precipitin ring. Electrophoretic patterns were obtained with Spinco Model R paper electrophoresis system. Duplicate strips were stained with bromphenol blue for protein and sudan black B for lipid. The absorbance of lipoprotein solutions was measured with a Coleman Junior spectrophotometer at 430, 460, and 490 mμ against a distilled water blank. A standard S_f 3-9 lipoprotein fraction containing 1 mg of protein/ml was used in aging, freezing and thawing, and heating experiments. This lipoprotein solution was dialyzed against 15 volumes of 0.15 *M* NaCl containing no ethylenediaminetetraacetic acid (EDTA). In aging studies, S_f 3-9 lipoprotein fractions were stored at 4°C, then titrated at stated intervals. Lipoprotein aliquots were stored with and without aqueous merthiolate, added to a final dilution of 1:10,000, as a preservative. Two procedures were used in freezing and thawing experiments. Procedure I: Five ml of the S_f 3-9 lipoprotein fraction was frozen at –20°C in the deepfreeze and then thawed immediately at 37°C in a water bath. Aliquots were removed for analysis before the first and after first, fifth, and tenth freeze-thaw cycles. Procedure II: Aliquots of an S_f 3-9 lipoprotein fraction, 2.5 ml, were frozen at –40°C in a dry ice-acetone bath, then thawed immediately at 37°C in water bath. Samples were removed for analysis after first, fifth, and tenth freeze-thaw cycles. An S_f 3-9 lipoprotein fraction was warmed at 56°C in water

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TABLE I. Effect of Freezing and Thawing on Titer of S_f 3-9 Lipoprotein Fraction Using Precipitin Ring Test.

No. of cycles	Titer Method I	Method II
0	1:64	1:64
1	"	1:128
5	1:32	1:64
10	1:64	1:128

bath for the heating studies. Samples were withdrawn after 15, 30, and 45 minutes for analysis.

Results. Preliminary aging studies indicated that S_f 3-9 lipoproteins, stored without added merthiolate, lost their specificity toward both S_f 3-9 and S_f 10-400 antisera in precipitin ring and agar diffusion tests after 6 to 8 weeks storage at 4°C. Staining revealed that lipoprotein solutions were grossly contaminated with microorganisms. When S_f 3-9 lipoprotein fractions were isolated and stored aseptically or aqueous merthiolate to final dilution of 1:10,000 was added to the lipoprotein fractions, there was no loss of titre and no bacterial contamination during 8 months storage at 4°C. An S_f 3-9 lipoprotein fraction was frozen and stored at –20°C for 7 months with no loss of antigenicity in precipitin ring titrations.

Ten freeze-thaw cycles did not affect the titre (Table I), agar diffusion pattern, or paper electrophoretic pattern of an S_f 3-9 lipoprotein fraction. However, absorbance of the lipoprotein fraction and presumably lipoprotein denaturation increased with freezing and thawing (Table II). Heating at 56°C for 45 minutes did not alter antigenicity, agar diffusion pattern, paper electrophoretic pattern, or absorbance of S_f 3-9 lipoprotein fractions.

Discussion. Low density lipoproteins are probably destroyed by bacterial action when lipoproteins are stored at 4°C. Since lyophilization or freezing will alter the physical properties of the lipoprotein fraction, aqueous merthiolate may be added to lipoprotein solutions to improve their storage for several weeks employed in chemical and physical investigations. Although freezing and thawing increased absorbance of a lipoprotein solution (indicating lipoprotein aggregation and denaturation), the immunological properties and paper electrophoretic pattern were unchanged. Furthermore, heating at 56°C, a procedure used to inactivate complement, did not alter the immunological properties of lipoprotein fractions. Thus lipoprotein lability does not affect immunological studies with low density lipoproteins utilizing agar diffusion pattern and precipitin ring technics. The ability to fix complement and immunodiffusion constants are altered when high density lipoproteins are aged and denatured(6). Complement fixation and immunodiffusion are, therefore, sensitive procedures for measuring lipoprotein denaturation, while precipitin methods are suitable for estimating lipoprotein concentration(7,9).

TABLE II. Effect of Freezing and Thawing on Absorbance of S_f 3-9 Lipoprotein Fraction.*

No. of cycles	Absorbance 430 mμ	460 mμ	490 mμ
0	.269	.269	.211
1	.269	.270	.215
5	.289	.285	.228
10	.315	.309	.249

* Lipoproteins frozen at –40°C and then thawed immediately at 37°C.

Summary. Human low density S_f 3-9 lipoproteins can be frozen and thawed, stored at –20°C, and heated to 56°C for 45 minutes without altering their immunological characteristics in precipitin ring and agar diffusion tests. Lipoprotein fractions stored at 4°C are decomposed by bacterial action. Aqueous merthiolate added to final dilution of 1:10,000 prevents bacterial contamination in lipoprotein fractions stored at 4°C without altering immunological characteristics of the lipoproteins. Freezing and thawing increases turbidity of lipoprotein solutions but does not alter lipid and protein patterns obtained with paper electrophoresis.

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Protection Afforded Mice Against Tourniquet Trauma Induced Mortality By Ganglion Blocking Drug Chlorisondamine Dimethochloride. (25095)

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Wiggers and co-workers(1) reported that dogs were afforded significant protection against lethal effects of severe hemorrhage by preadministration of the adrenolytic drug dibenamine. They postulated that this protection was due to improved circulation in those tissues whose circulation is usually most severely curtailed as a result of sympatho-adrenal activation, which is a generalized reaction to severe hemorrhage or trauma. Many reports have since described the significant protection afforded experimental animals against lethal effects of traumatizing and/or hemorrhagic procedures, by *pre*-administration of one or more adrenolytic, sympatholytic and/or ganglion blocking drugs. References in 3 of these papers(2,3,4) include most of the literature. Some reports include data on mortalities when the test drug was administered after severe trauma or hemorrhage. The common finding has been failure of the drug to afford test animals significant protection when so administered. In tourniquet trauma, precipitation of the shock syndrome does not occur until after removal of tourniquets. Hence in this type of trauma it should be possible to assure arrival of drug at postulated vital centers before precipitation of the shock syndrome, by lengthening the period of occlusion of circulation to the hind legs, to permit injection of the drug prior to removal of hind leg ligatures. Alternatively, the innate severity of the shock syndrome might be minimized somewhat, at least in theory, by removing the ligatures earlier, and injecting the drug after such removal, when it would presumably be less capable of combating shock. Experiments were therefore initiated with the ganglion blocking drug, chlorisondamine dimethochloride (CD), with the above relationships in mind. While the original problem has not been resolved, our experiments revealed a protective capability of the drug (CD) against tourniquet trauma induced mortality, injected at time of ligature removal, sufficiently great to warrant this report.

Methods. Mice used were Carworth Farms males, about 3 months of age and 20-30 g body weight. Tourniquet trauma was inflicted by occluding circulation to both hind legs for about 2 hours using rubber bands(5). Control mice were injected with water, test mice with an aqueous solution of chlorisondamine dimethochloride. Other procedures are indicated in Table I.

Results. Table I gives data for 2 experiments, performed in the same manner, with similar results. The drug dose (5 mg/kg) was one which earlier experiments indicated would significantly increase the likelihood that mice would survive tourniquet traumatization, when the drug was injected at time of ligature removal. Experiments of Table I were designed to test whether degree of protection was measurably altered when the drug was administered 15 minutes, rather than zero minutes, after removal of hind leg ligatures. Since it was considered essential that the time between application of ligatures and injection of drug or vehicle (water) be kept constant (at 2 hours), this necessarily resulted in a ligation period of 120 minutes for mice of Groups A_1

TABLE I. Protective Action of Chlorisondamine Dimethochloride (Ciba Ecolid) against Tourniquet Trauma Induced Deaths of Mice.

Group	Min. before (–) or after (+) removal of hind leg ligatures: Ligature application	I.P. inj. of water or drug	Drug dose (mg/kg)	No. of mice*: No. traumatized	No. dead to designated hr after ligature removal: 6	24	48	120
A_1	–120	0	Zero	45	10	26	32	32
A_2	–105	+15	"	45	9	27	28	30
$A_1 + A_2$				90	19 (21%)	53 (59%)	60 (67%)	62 (69%)
B	–120	0	5	64	1 (2%)	20 (31%)	27 (42%)	29 (45%)
C	–105	+15	5	65	1 (2%)	26 (40%)	32 (49%)	36 (55%)
Probabilities that observed differences were due to chance, as estimated by Chi Square procedures (NS, p over .05, not significant)								
A *vs* B:					<.01	<.01	<.01	<.01
A *vs* C:					"	.04	.04	NS
B *vs* C:					NS	NS	NS	"

* Carworth Farms males, 20-30 g, about 3 mo of age.

and B, and of 105 minutes for mice of Groups A_2 and B. This alteration in ligation period did not result in an appreciable difference in mortality rate as between the 2 control groups, A_1 and A_2. Hence data for these 2 Groups have been summed for use in estimation of probabilities by the Chi Square method. Since Degrees of Freedom = 1, Yates' 0.5 correction was used in calculation of Chi Square values(6). If a probability of 0.01 or less was used as criterion of significance, the present data indicate that for mice the likelihood of indefinite survival following tourniquet traumatization was significantly increased by administration of chlorisondamine dimethochloride at time of ligature removal, whereas this was not the case for the drug administered 15 minutes after ligature removal. While the data are not in disagreement with the hypothesis that delay in injection of drug is deleterious to survival, they are inadequate to demonstrate this relation. The change in mortality rate associated with the delay is apparently small; hence very large numbers of mice would be needed to demonstrate unequivocally whether the delay was deleterious.

Discussion. Chlorisondamine dimethochloride is a drug with relatively prolonged ganglion blocking effects which begin shortly after its administration(7). Data here presented suggest that procedures might be developed whereby some drugs of this nature would be usefully employed clinically in certain traumatic situations. However, these situations should be very clearly understood and carefully defined and circumscribed. In experiments performed for other purposes we noticed repeatedly that mice preinjected with chlorisondamine dimethochloride are much more susceptible to lethal effects of severe cold or high insulin dosage than control mice. We reported(8) that while chlorpromazine had protective action against both heat and tourniquet trauma induced mortality, if administered well before traumatization, this drug actually decreased the likelihood that mice would survive heat trauma if administered immediately after traumatization. The general impression from the literature is that for most traumatic situations and most drugs, post-administration of adrenolytic or ganglion blocking drug does not increase but may actually decrease likelihood of survival.

Summary. Survival-mortality data were obtained for tourniquet traumatized, adult Carworth Farms male mice for 120 hours after zero hour (time of removal of both hind leg ligatures). Higher percent survivals were noted throughout for test mice injected intraperitoneally with chlorisondamine dimethochloride, 5 mg/kg, than for control traumatized mice injected with water. The p (proba-

bility) values for test *vs.* control mice were: (A) for mice injected at zero hour, 0.01 or less at 6, 24, 48 and 120 hours thereafter, and (B) for mice injected 15 minutes after zero hour, 0.01 or less ONLY at 6 hours thereafter.

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Normal and Experimental Growth of Rat Mammary Gland.* (25096)

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It is generally accepted that estrogenic hormones stimulate growth of mammary gland duct system while estrogen and progesterone stimulate lobule-alveolar growth in intact and ovariectomized rats. Many investigators have attempted to determine levels of these hormones which produce optimal mammary gland growth(1). Differences in extent of lobule-alveolar growth have been evaluated by visual examination of whole mounts of glands. Kirkham and Turner(2) first suggested the use of desoxyribonucleic acid (DNA) content as an objective index of mammary gland cellular growth. Griffith and Turner(3) have shown that mean DNA content/nucleus of rat mammary gland remained constant during pregnancy. Determination of total mammary gland DNA permits quantitative estimation of gland growth during normal pregnancy and following growth under experimental conditions. In the present study, frequency distribution of total mammary gland DNA of rats pregnant 18-20 days has been compared with that of adult ovariectomized rats treated with 1 μg estradiol benzoate in synergism with graded levels of progesterone for 19 days.

Materials and methods. Groups of pregnant and ovariectomized rats of Sprague Dawley-Rolfsmeyer strain with initial weight of 220-270 g were used. Animals killed 18-20 days of pregnancy and ovariectomized rats killed 34 days postcastration served as controls. Remaining ovariectomized rats received daily subc. injections of 1 μg estradiol benzoate (EB) alone and with 1-10 mg progesterone (P) for 19 days commencing 14 days after ovariectomy. They were killed one day after last injection, skinned rapidly and abdominal-inguinal glands removed and frozen for 4 days. Tissues then were thawed and fat extracted in boiling 95% ethanol for 6 hours followed by ether extraction for another 6 hours. DNA was extracted from 25 mg aliquots of finely ground, dry, fat-free tissue (DFFT) by the method of Schneider(4) with exclusion of fat extraction and cold trichloroacetic acid extraction steps. DNA was determined according to method of Webb and Levy(5). The product of the quantity DNA/mg DFFT and DFFT, expressed as unit body weight, was estimated as total DNA/posterior 6 glands. Visual observations were made for presence of lobule-alveolar development but

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TABLE I. Mammary Gland DNA of Adult Rat Ovariectomized 14 Days Followed by 1 μg Estradiol Benzoate (EB) and 1-10 mg Progesterone (P) for 19 Days.

Treatment (amt/day)	No. of rats	Body wt (g)	DFFT* (mg/100 g)	DNA/mg DFFT (μg)	Total DNA posterior 6 glands (mg/100 g)
Ovariectomized controls	15	273.7	132.4	23.20 ± .54‡	3.05 ± .14[1]
EB alone	5	246.8	197.0	22.86 ± 1.27	4.48 ± .15[2]
EB + 1 mg P	5	254.8	210.1	27.30 ± 1.10	5.71 ± .23[3]
+ 2 "	20	291.4	188.4	39.33 ± .67	7.41 ± .31[4]
+ 3 "	19	269.0	219.0	35.65 ± 1.09	7.72 ± .25
+ 4 "	21	274.6	219.0	35.70 ± 1.01	7.72 ± .23
+ 5 "	21	281.2	235.3	33.17 ± 1.22	7.63 ± .28
+ 6 "	10	278.4	225.9	35.50 ± 1.53	7.95 ± .34
+ 7 "	5	279.6	264.6	33.73 ± .74	8.87 ± .55[5]
+ 8 "	5	272.4	225.7	36.18 ± .63	8.16 ± .60
+ 9 "	5	267.4	226.1	37.29 ± 2.41	8.43 ± .56
+ 10 "	5	280.8	219.0	41.01 ± 1.20	8.96 ± .36[6]
Pregnant controls	19	324.3†	230.9	33.24 ± .84	7.63 ± .40
Aggregate, EB + 2-10 mg P	111	278.3	219.9	36.09 ± .45	7.83 ± .12

* Dry, fat-free tissue.
† Corrected for wt of fetuses.
‡ Stand. error of mean.

Student's "t"	Probability
1-2	.001
2-3	.005
3-4	.025
4-5,6	.05

no attempt was made quantitatively to determine the extent of growth.

Results. Results of daily administration of EB alone and with graded doses of P for 19 days are presented in Table I. Total DNA of glands of ovariectomized rats receiving EB alone was significantly greater than that of ovariectomized controls. Administration of EB and 1 mg P increased total DNA above that of EB alone and upon increasing the P level to 2 mg, a further increase in total DNA was obtained. Treatment of rats with EB and 3-10 mg P did not significantly increase total DNA above that resulting from administration of EB and 2 mg P. Mean total DNA of glands of rats receiving EB and 2-10 mg P did not differ significantly from that obtained with rats pregnant 18-20 days. DNA values of experimentally developed glands (2-10 mg P levels) were grouped to determine the frequency distribution. Coefficient of skewness (g_1) of DNA values obtained with pregnant rats and those of rats receiving EB and 2-10 mg P were .02 and .14, respectively. Since g_1 values did not differ significantly from a g_1 of zero, DNA values of both pregnant and experimentally treated animals fell within a normal frequency distribution (Fig. 1). Analysis of data also indicated a highly significant correlation (r = .74) between body weight and DFFT weight.

Visual examination of mammary glands of rats ovariectomized 2 weeks, indicated an atrophic duct system with occasional end buds but no alveoli. EB treatment resulted in increased duct growth and end-bud formation. Rats receiving EB and 1-10 mg P exhibited lobule-alveolar growth. The extent of growth, however, was not rated.

Discussion. Numerous investigators have attempted to determine the synergistic level

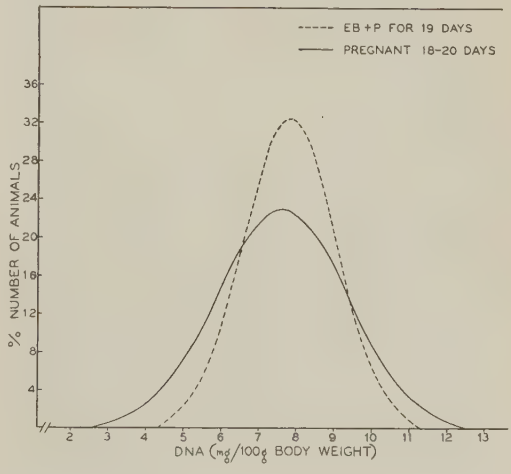

FIG. 1. Frequency distribution of DNA of rat mammary gland. EB + P – 1 μg estradiol benzoate + 2-10 mg progesterone.

of estrogen and progesterone which would produce optimal mammary gland growth in the rat(6-11). Results, however, have been conflicting due in part to age of animals used, interval and duration of treatment and difficulties encountered in visual estimation of extent of growth.

Data of present study indicate daily administration of 1 μg EB and 2 mg P for 19 days resulted in mammary gland growth comparable to that of rats pregnant 18-20 days. Increasing the P level to 10 mg daily had little, if any, further beneficial effect upon growth. The highly significant correlation between body weight and weight of DFFT appears to implicate body weight as a contributing factor in estimation of total DNA of the rat mammary gland. Harkness and Harkness(12) have shown that collagen content and presumably amount of connective tissue of the rat mammary gland do not change during pregnancy and that collagen content is relatively the same between animals when expressed as unit body weight. Since connective tissue possesses relatively fewer cells than glandular tissue/unit mass and, therefore, less DNA, it appears that differences in body weight of animals under similar conditions reflect greater differences in weight of DFFT rather than in quantity of DNA. Total DNA calculated from quantity DNA/mg DFFT and total DFFT, expressed as unit body weight (DFFT/100 g), appears, therefore, to be a more reliable index of total cellular proliferation of mammary tissue.

The lower standard error (Table I) and decreased range (Fig. 1) of DNA values of experimentally developed glands indicate decreased variability in gland growth as compared with that of pregnant rats. The decreased variation may result from administration of EB and P to animals which do not normally secrete these hormones in quantities necessary for optimal gland growth. Variation in total DNA/100 g of animals under the influence of optimal amounts of EB and P may be due, in part, to inherent variation in responsiveness of mammary epithelial cells to ovarian hormones. On the other hand, much of the variability observed may result from variation in endogenous secretion rate of thyroxine(13), growth hormone(1), glucocorticoids(1), etc., which synergize with ovarian hormones in stimulating mammary gland growth. Determination of mammary gland DNA affords a method whereby the synergistic action of these hormones alone and in combination, upon variation in total gland growth may be studied.

Summary. 1) Daily administration of 1 μg estradiol benzoate (EB) and 2 mg progesterone (P) to mature ovariectomized rats for 19 days resulted in mammary gland growth (mean total DNA/100 g) comparable to that of rats pregnant 18-20 days. Increasing the progesterone level to 10 mg daily has little further beneficial effect on mammary gland growth. 2) Variability in growth of experimentally developed gland was less than that of pregnant controls. 3) Total DNA appears to be a more reliable index of mammary gland growth when expressed as unit body weight.

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Effect of Hypophysectomy and Adrenalectomy in Nucleoside-Induced Nephrosis in the Rat. (25097)

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The effectiveness of aminonucleoside of puromycin* in production of a nephrotic-like syndrome in rats, has been reported and details(1,2,3) and timing of the syndrome have been well described. Experiments reported here were undertaken less from the point of view of the clinical syndrome than the effects observable in renal tissues. The criteria adopted, therefore, were those conventionally employed in examination of stained sections. Our purpose was to compare tolerance of rat kidney to nucleoside-induced nephrosis under 3 conditions: 1) in intact animal, 2) in hypophysectomized and 3) in adrenalectomized animal. Immature rats of Sprague-Dawley strain were used. Group I consisted of rats purchased hypophysectomized; Group II were left intact and Group III were adrenalectomized in our laboratory. Each of the latter group received Meticortelone,* 5 mg subcutaneously, at time of surgery. No further hormone replacement was given in any group. All animals were given complete rations, ground. Groups I and II were given tap water; Group III was given 0.9% saline. Aminonucleoside was administered in doses of .01 mg/g body weight by subcutaneous route, for 10 days. All were sacrificed 48 hours after end of the course. Kidney slices were made immediately; all autopsy material was fixed in usual manner, in 10% formalin.

Observations. All animals had clinically detectable ascites by the 8th day. All had massive proteinuria. Group III showed far more interstitial edema than did Group I and Group II, and the large amounts of fluid contained in the peritoneal and pleural cavities had a fibrinous appearance in Group III which was not noted in the other groups. Ascitic fluid was taken wherever feasible, upon which determinations of sodium and of creatinine were made. These data were noncontributory.

Observations made on stained sections were in general agreement with those already published, in the case of Group II. Here, architecture of the kidney was disrupted by patchy areas of recent interstitial fibrosis and edema, and by many dilated distal tubules filled with hyaline casts, such casts being particularly prominent in the collecting ducts. Few changes were noted in the glomeruli. A large amount of amorphous material, presumably protein precipitate, was visible in tubules at all levels. Fragmentation of cytoplasm and sloughing of tubular epithelium were notable, particularly around the casts.

In adrenalectomized animals (Group III), these changes were considerably less marked.

In hypophysectomized animals (Group I), renal elements were normal.

Variations in concentrations of sodium and creatinine could hardly be considered significant, in the limited number of specimens comprising this pilot study.

Conclusions. Experimental nephrosis induced in hypophysectomized immature rat is clinically similar to the syndrome seen in the

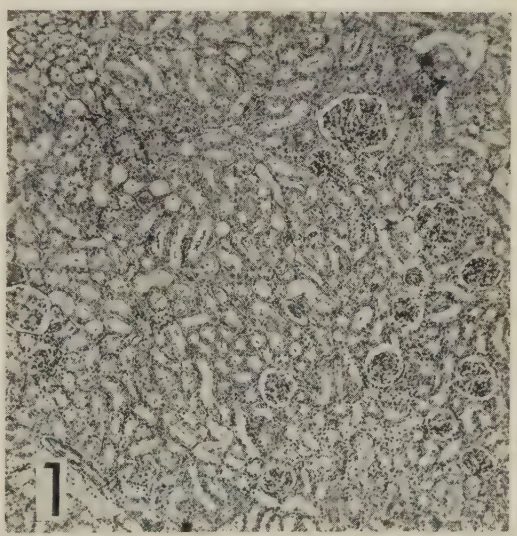

FIG. 1. Kidney from hypophysectomized rat. H & E × 73.

* Kindly supplied by Lederle Labs.

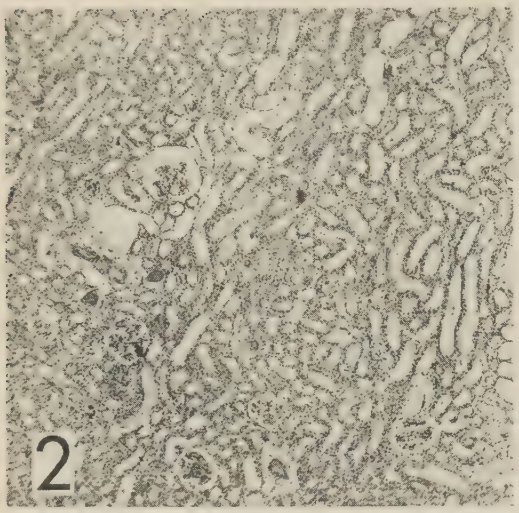

FIG. 2. Kidney from intact rat. H & E × 55.

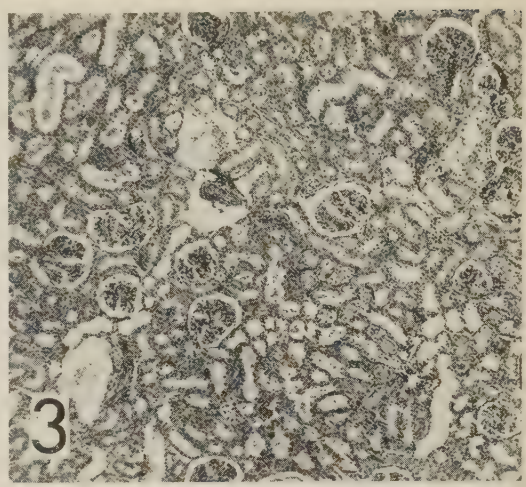

FIG. 3. Kidney from adrenalectomized rat. H & E × 55.

intact animal. Evidences of renal damage in intact animals, however, were not present in these which had previously undergone hypophysectomy. Pathological changes which occurred in kidneys of the adrenalectomized group were similar to those described in normal rats, but less severe. Whether for direct or indirect reasons absence of functioning hypophysis was associated with tolerance of renal tissue toward aminonucleoside even though the nephrotic syndrome was clinically similar.

Summary. Nephrosis was induced in immature rats by subcutaneous administration of aminonucleoside. Subjects were divided in 3 groups: I, hypophysectomized, II, intact and III, adrenalectomized. All 3 groups developed proteinuria and ascites. Examination of stained sections, however, showed that kidneys of intact rats had undergone marked deterioration. Pathological changes were less marked in the adrenalectomized group, and absent in the hypophysectomized group.

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Significance of Nystatin Uptake for Its Antifungal Action. (25098)

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Nystatin, a polyene antifungal agent produced by *Streptomyces noursei*(1), inhibits growth and utilization of various substrates by fungi, especially yeasts(2). It was previously observed(3) that nystatin is removed from solution by organisms whose growth is sensitive to its action, but not by insensitive organisms. The properties of this uptake appeared to correlate with available information both on specificity of nystatin and on its fungicidal and metabolism-inhibiting actions. It was suggested, therefore, that this uptake is essential for nystatin action and is a critical factor in determining sensitivity. To evalu-

ate more precisely the importance of absorption of nystatin, we examined a series of sensitive : resistant pairs of *Candida* strains(4,5). We have also studied the effect of agents which alter uptake of nystatin by yeast and the consequent alteration of its fungicidal and metabolic action. Sterols(6), bile salts(7), and high concentrations of phosphate(5) have been reported to reduce biological effectiveness of nystatin or related polyene antifungals. The relation of certain of these agents to the absorption process has also been examined. A separate publication concerning effect of sterols on action of several polyene antifungal antibiotics is in preparation.

Methods. Absorption tests were performed as previously described(3). Unless otherwise stated, nystatin was dissolved in 0.2 ml of 1% aqueous dimethyl sulfoxide. To this volume were added the appropriate buffer, 5 mg dry weight of *Saccharomyces cerevisiae* or *Candida* strains and water to 6 ml. The buffers were 0.067 M pH 4.0 phthalate or pH 6.8 to 7.0 phosphate(8), or a 0.017 M succinate-0.017 M phosphate buffer at pH 4.5. The tubes were incubated with occasional shaking at 30°C, and after periods indicated were chilled and centrifuged at 2500 x g for 15 minutes. Absorbance at 321 mμ was used as a measure of nystatin content of supernatant fluids. In certain experiments the absorption tests were performed in Warburg flasks to permit a better comparison with metabolic measurements. Absorption is more rapid under these conditions, perhaps because there is better contact between nystatin and cells. However, the basic characteristics of the absorption are unchanged. Manometric studies were carried out at 30°C, using standard Warburg technics. For measurement of oxygen uptake, the gas phase was air. Unless otherwise stated, each cup contained phthalate buffer, pH 4, 2.5 mg dry weight of cells (washed 4 times in water), the antifungal agent in 0.1 ml of 1% aqueous dimethyl sulfoxide, 60 μM of glucose, and water to 2.8 ml. The center well contained 0.2 ml of a 10% KOH solution. For anaerobic studies the gas phase was 95% N_2 and 5% CO_2. Unless otherwise stated, nystatin, glucose, zymosan, serum albumin, or inhibitors were added at 0 minutes. The nystatin was either a highly purified crystalline preparation with biological activity(9) of approximately 5500 units/mg (Squibb Lots HV-917 or HV-942), or the commercial crystalline product with biological activity of 2850 units/mg (Lot 15289-011). These products and the methyl ester and N-acetyl derivative of nystatin were kindly furnished by Dr. J. D. Dutcher, Squibb Institute for Medical Research. The methyl ester and N-acetyl derivative have no antifungal activity either *in vivo* (oral dosage) or *in vitro* (Dutcher, J. D., *et al.*, unpublished observations). Zymosan was washed 4 times and resuspended in water before use. An analysis of this preparation (No. 6B14) is given by Di Carlo and Fiore(10). The sensitive : resistant pairs of organisms described by Littman *et al.*(4) were generously provided by Dr. J. Pagano, Squibb Institute. Dr. S. G. Bradley, University of Minnesota, furnished the *Candida stellatoidea* strains(5). These cells were grown overnight at 28°C with shaking in Penassay broth (Difco) plus 1% glucose. The cells were washed 4 times in distilled water and resuspended in water. The *S. cerevisiae* was commercial bakers' yeast from Anheuser-Busch. The term yeast, when not further defined, refers to this material.

Results. The time sequence for action of nystatin on metabolism is significant, since it affords both an indication of the manner in which nystatin acts and of the changes expected when uptake of nystatin is prevented or reduced. Fig. 1 presents a typical test, using inhibition of glycolysis as the measure of nystatin action. Analogous results are obtained if respiration is the criterion. It should be noted that the rate of glycolysis is normal or even elevated until a sudden drop in activity occurs. Within a short period, gas production generally ceases completely. It has not been possible to reverse this loss of activity once it has occurred. Although higher concentrations of nystatin are more likely to cause an initial stimulation than are lower levels, the primary effect of concentration is to determine the time required before the sharp drop in activity takes place. There is also a rough inverse proportionality between time required for inhibition and quantity of

TABLE I. Effect of Nystatin on Sensitive : Resistant Pairs of Candida Strains.*

Organisms	Ratio of fungicidal conc., R/S†	Conc. of nystatin inhibiting glucose oxidation			Absorption of nystatin (10 μg/ml) in 60 min.		
		S	R	R/S	S	R	R/S
		μg/ml					
Candida guilliermondii	>4 ‡	3	16	5	5.1	1.3	1:4
C. parakrusei	1.6‡	9	13	1.4	2.8	2.1	1:1.3
C. tropicalis	13.3‡	2	8	4	5.4	3.5	1:1.5
C. stellatoidea	12 §	1.3	8	6	5.4	3.6	1:1.5

* The commercial nystatin preparation and pH 4.5 succinate-phosphate buffer were used in all tests.

† Ratio of values for resistant strain (R) and for parent sensitive culture (S).

‡ Data of Littman *et al.*(4).

§ Data of Bradley(5).

nystatin absorbed by the cells.

If nystatin uptake is essential to its action, an agent which reduces or delays this uptake should extend the period during which metabolism appears normal, and thus will appear to furnish complete protection during this time. If inhibition of uptake is not complete, a rapid loss of metabolic activity will eventually occur and cannot then be reversed. These general principles will be used in assessing the experiments which follow.

Table I summarizes comparative tests with pairs of nystatin-resistant : sensitive strains of *Candida*. From the resistant : sensitive ratios presented, it can be seen that the reported resistance of these strains(4,5) to fungicidal and fungistatic action of nystatin was paralleled by increased resistance of their oxidative metabolism. The concentrations of nystatin which inhibit oxidation approximated those required to prevent growth, as observed previously(3). The resistant strains all absorbed less nystatin than did comparable sensitive strains, but there was no quantitative correlation between magnitude of changes in uptake and in sensitivity. For example, differences in uptake with *C. tropicalis* and *C. stellatoidea* appear too small to account for the changes in sensitivity.

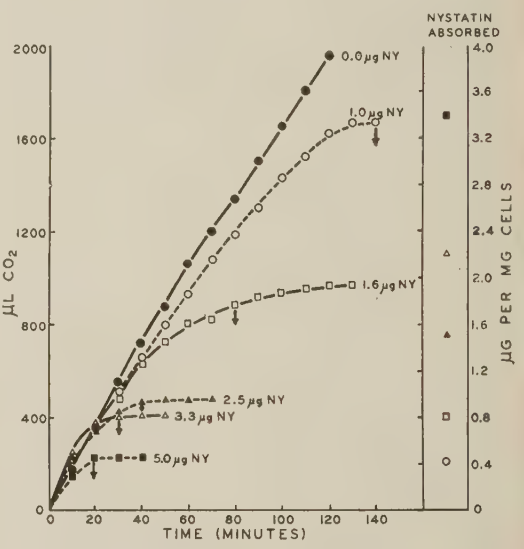

FIG. 1. Action pattern of nystatin (NY) on glycolysis. Final volume = 3.0 ml. Purified nystatin used; values on graph represent μg/ml. At the arrow (↓) metabolism is considered to have ceased. Amount of nystatin absorbed in 30 min., as determined in parallel series of vessels, is represented in column at right (symbols as in graph of CO_2 production).

TABLE II. Glycolysis and Absorption of Nystatin by *S. cerevisiae* in the Presence of Serum Albumin.

Nystatin,* μg/ml	Serum albumin, mg/ml	CO_2 production, μl/60 min.	Metabolic activity ceased, min.	Nystatin absorbed, μg/mg yeast 60 min.†
0	0	990	>120	
5	0	65	15	4.6
0	32	1040	>120	
5	12	435	35	2.0
5	19	540	45	1.0
5	32	810	75	.4
21	32	81	20	

* Purified preparation.

† Absorption essentially complete at 20 min.

High levels of human serum albumin decreased absorption of nystatin by yeast and protected glycolysis against its inhibitory action (Table II). This protection approximates that expected on the basis of reduction in nystatin uptake (Fig. 1).

Zymosan, a yeast fraction shown by Di Carlo and Fiore(10) to consist primarily of yeast cell ghosts, is a potent binding agent for

TABLE III. Effect of Zymosan on Glycolysis by *S. cerevisiae*.

Nystatin* added, μg/ml	Zymosan, mg/ml	μl CO_2/60 min.	Metabolism ceased, min.	Residual nystatin at 10 min., μg/ml†
0	0	910	>120	0
3.5	0	210	25	3.5
"	1.1	264	25	2.7
"	3.5	815	65	1.4
"	10.7	965	>120	.4

* Purified nystatin used. Buffer was pH 4.5 succinate-phosphate.

† Parallel test series without cells to indicate magnitude and rapidity of removal of nystatin by zymosan.

nystatin. Yeast glycolysis can be protected by zymosan and this protection can be explained readily by rapid removal of nystatin by the zymosan (Table III). Neither zymosan nor serum albumin will reverse the action of nystatin once inhibition of metabolism has occurred.

It was of interest to know if biologically inactive derivatives of nystatin would be absorbed by yeast. It was found that amounts of methyl ester and N-acetyl derivative absorbed by intact yeast and zymosan at pH 4.0 or 6.8 were much less than the uptake obtained with nystatin itself. Values for absorption were generally within experimental error of procedure. A definite uptake—approximately 25% of nystatin absorbed under the same conditions—was demonstrated only with methyl ester-intact yeast combination at pH 4.0.

Bradley(5) reported that 0.5 M phosphate prevented partial inhibition of oxidation in *C. stellatoidea* which he obtained with 20 μg of nystatin/ml. This concentration of phosphate did inhibit uptake of nystatin by yeast by approximately 60% under our conditions; however, it is toxic to glucose oxidation by *S. cerevisiae* and actually increased its sensitivity to nystatin (unpublished data). Thus no direct comparison between our results and those of Bradley can be made.

Schneierson *et al.*(7) observed that addition of 1% bile salts to the medium reduced sharply the activity of nystatin in inhibiting growth of *C. albicans*. The commercial preparation of bile salts used by these authors interfered in our optical test for binding. Purified sodium desoxycholate could be tested at pH 7 (precipitation at pH values below 6), and under these conditions reduced nystatin uptake by 80 to 90%. Since bile salts are known to form inclusion complexes(11), one may suggest that they reduce the effective concentration of nystatin and thus reduce its activity without direct metabolic interaction.

Uranyl ions are known to combine with the surface of intact yeast but not to penetrate the cell. They inhibit uptake of sugars by yeast(12). At 1 x 10^{-4} M, uranyl acetate inhibited uptake of nystatin by 60 to 80%. This concentration reduced the rate of oxidation of glucose by yeast but effected almost complete protection of residual oxidation against nystatin (Table IV).

The effect of dinitrophenol and of arsenate

TABLE IV. Effect of Uranyl Acetate, Sodium Arsenate, and Dinitrophenol on Metabolism of *S. cerevisiae*.

Test material*	Nystatin,† μg/ml	μl O_2 consumption 60 min.	μl O_2 consumption 120 min.	Metabolic activity ceased, min.
Control	0	585	1100	>120
	3.5	70	70	25
	5	115	115	15
Uranyl acetate, 1 × 10^{-4} M	0	304	580	>120
	3.5	350	465	120
Sodium arsenate, 2 × 10^{-2} M	0	170	345	>120
	5	200	310	110
Dinitrophenol, 2 × 10^{-4} M	0	450	940	>120
	5	310	360	45

* Uranyl acetate added 20 min. before glucose and nystatin; other test materials added with glucose and nystatin at 0 min.

† Purified preparation.

was investigated, since Halvorson *et al.*(13) reported that the former, but not the latter, inhibits uptake of amino acids into the amino acid pool of yeast. At the concentration employed by these investigators (2×10^{-4}M), dinitrophenol was relatively ineffective in reducing absorption of nystatin (inhibition 20% or less) and provided only a short protection of oxidation (Table IV). At a 2×10^{-2}M concentration, arsenate inhibited nystatin uptake ca. 60%, and this effect was not prevented by 6.7×10^{-2}M phosphate. Arsenate did not produce a significant stimulation of endogenous respiration. It reduced glucose oxidation sharply, and effectively protected the residual respiration against nystatin for the period of the test. With a 8×10^{-2}M arsenate concentration, oxidation was inhibited almost completely, whereas 4×10^{-3}M did not produce significant protection. For inhibition of nystatin uptake to occur, arsenate must be present during contact between nystatin and yeast, since cells incubated with arsenate for 30 minutes and then washed retained their ability to absorb the usual amounts of nystatin. When yeast was incubated with nystatin (5 μg/ml) until oxidation of glucose had ceased, addition of arsenate did not restore activity. Thus the action of arsenate appears to be preventive only.

Discussion. The results lend considerable support to the proposal that nystatin uptake by cells is critical for its inhibitory action(3). The protective action of the various agents for metabolism correlates well with conditions under which they absorb significant amounts of nystatin or reduce rate or extent of its absorption by yeast. The characteristic of this protection is increase in time before a sharp drop in metabolic activity occurs. This pattern was predicted from the time sequence of nystatin action illustrated in Fig. 1. However, of 4 resistant strains tested, 2 had changes in sensitivity greater than one would anticipate from the small reductions in amount of nystatin they absorbed. Thus, factors other than uptake must also be important in determining sensitivity to nystatin.

The kinetics of metabolic inhibition by nystatin are compatible with the concept that nystatin initiates a destructive action on critical cell constituents. Scholz *et al.*(14) have shown that nystatin produces a rapid decrease in amounts of glycolytic enzymes and protein extractable from the yeast cell. It is probable that these enzymes are initially present in excess, so that no effect on glycolysis is observed in our experiments until the enzymes become rate-limiting. Concentration of nystatin would determine rate of this destruction and thus the time until metabolic changes become evident.

Previous work(2) has shown that uptake of nystatin is sharply increased at acid pH. The apparent pK_a of the group concerned approximates that of the carboxyl of nystatin. The present finding that the methyl ester and N-acetyl derivatives are not absorbed to a significant extent allows one to suggest that the ionic species of nystatin absorbed by yeast is HOOC . . . NH_3^+.

Summary. 1) Time sequence for inhibition of yeast respiration and glycolysis by nystatin is described and its significance is discussed. 2) A series of nystatin-resistant : sensitive pairs of *Candida* strains was examined. With various pairs, resistance of growth to nystatin was accompanied by similar resistance of metabolic activity to the agent. Resistant strains absorbed less nystatin than did sensitive organisms under same conditions, but there was no quantitative correlation between sensitivity and absorption. Reduced uptake of nystatin is probably only one of the ways by which resistance can be achieved. 3) Addition of serum albumin, zymosan, uranyl ions, arsenate, or dinitrophenol could protect yeast metabolism in varying degrees against the action of nystatin. Inhibition, once obtained, could not be reversed. The protective action of various agents approximated that expected from their effect on absorption of nystatin by yeast. 4) The methyl ester and N-acetyl derivatives of nystatin, which do not inhibit growth or metabolism of fungi, were not absorbed to a significant extent by either fresh yeast or zymosan. Since absorption occurs primarily below pH 5, the ionic species HOOC . . . NH_3^+ is probably the material absorbed by yeast. 5) The results furnish additional evidence for the critical nature of up-

take of nystatin by microorganisms in determining sensitivity to its action.

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Effect of Urea on Enzyme Activity of the Choroid Plexus. (25099)

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Fremont-Smith and Forbes(1) demonstrated that urea when injected into peritoneum of the cat had a rather striking effect on reduction of intracranial pressure. They suggested its clinical application because of its lack of toxicity and its ready elimination by normal kidneys. Smythe, Smythe and Settlage(2) later found that 17% urea produced a profound and persistent drop in intracranial pressure. On this basis, Javid and Settlage(3) reported effective use of a 30% solution of urea in reducing the intracranial pressure of 21 patients. No secondary rise was found as had been the experience with hypertonic solutions of sodium chloride and dextrose. The effect of urea in reducing intracranial pressure is present despite bilateral nephrectomy in the monkey(4). The specific role of the choroid plexus in formation of cerebrospinal fluid is not understood. To gain insight into the role of this organ, the activity of 7 representative enzymes has been measured in the choroid plexus of the cat(5). This tissue was approximately one-third to one-half as active as liver and kidney and more active than most areas of the brain in specific reactions. Our findings do not allow us to conclude that this structure is responsible for production of CSF, but its anatomical and enzymatic properties strongly suggest that it may play a role in elaboration of this fluid. Since urea has a profound effect upon intracranial pressure, it became of interest to study the effect of this drug upon selected enzymatic activities of the choroid plexus.

Materials and methods. All studies were done on the cat. The animals were anesthetized with intravenous nembutal, 30 mg/kg, and the animal placed in the prone position. The cisterna magna was entered with a fine 25 gauge needle attached to polyethylene catheter connected to a pipette clamped in horizontal position and graduated in hundredths of a milliliter. Observations of the CSF flow were made every 10 minutes. After urea had been given intravenously, observations were continued for 3 hours. At end of experiment, the animal was sacrificed, and a sample of blood, a kidney, and entire brain were removed. The choroid plexus along with

TABLE I. Biochemical Activity of Cat Tissues.

Tissue	Choroid plexus	Kidney	Blood	
Alkaline phosphatase	1767 ± 96 (26)†	3720 ± 804 (7)		μmoles p-nitrophenol phosphate, split/hr/g
Idem, after I.V. urea (1 g/kg body wt)	1311 ± 295 (8)	3763 ± 1001 (8)		*Idem*
Succinic dehydrogenase	5760 ± 380 (13)	18540 ± 1600 (8)		μl/hr/g
Idem, after I.V. urea	6863 ± 909 (3)	17567 ± 1689 (4)		*Idem*
Carbonic anhydrase	179* ± 12 (56)	100.1 ± 12.3 (12)	450 ± 9.0 (74)	Ashby units/g tissue
Idem, after I.V. urea	175* ± 15.8 (4)	141.8 ± 29.7 (4)	308 ± 74 (4)	*Idem*

* Corrected for blood.

† No. of animals in parentheses.

sample of kidney were then dissected and immediately studied for its metabolic activity. Three enzymes were selected: alkaline phosphatase, found in high concentrations in walls of blood vessels, and succinic dehydrogenase and carbonic anhydrase, found previously to be in high concentrations in the ependymal layer. Analysis of tissues for activities of alkaline phosphatase, succinic dehydrogenase, and carbonic anhydrase was carried out as described previously(5).

Results. Table I illustrates no major differences in activity of these enzymes of the choroid plexus and kidney under the influence of intravenous urea, despite the fact that this drug promotes reduction of CSF flow and presumably pressure in the cat since there was no hemo-dilution as measured by hemoglobin analysis. The marked reduction of blood carbonic anhydrase values after urea treatment cannot be accounted for. Reduction in CSF flow is comparatively shortlived (Fig. 1), and our experiments showed the original flow rate was resumed generally within 40 minutes although some cases might be inhibited in flow rate for as long as 80 minutes. Once the original flow rate has been reestablished, intravenous urea may again have the same effect on flow rate.

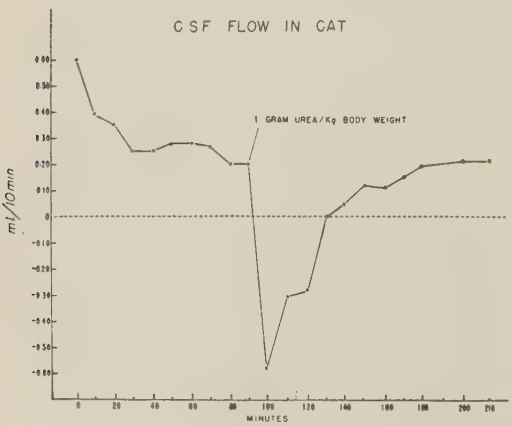

FIG. 1.

Discussion. Urea has been known to be a very effective diuretic but its effects in reducing intracranial pressure are not due to this phenomenon since bilateral nephrectomy in the experimental animal does not interfere with this effect in reducing pressure(4). Urea does not penetrate the blood-brain barrier as readily as it does other tissue(4) and it is conceivable that its effect may be due entirely to a difference of osmolarity between blood and cerebrospinal fluid. However, this study illustrates that enzyme activity of the choroid plexus is unaltered following intravenous urea therapy. From previous studies, however, we have been unable to conclude that CSF flow is dependent on enzymatic activity of the choroid plexus. It is quite possible that flow of CSF during urea therapy is unaltered and only absorption is changed.

Summary. Intravenous urea has been administered in cats. The flow rate of CSF is radically reduced but the activity of 3 enzymes of the choroid plexus (alkaline phosphatase, succinic dehydrogenase, and carbonic anhydrase) is unaltered.

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Blood Thromboplastin: Its Preparation and Properties.* (25100)

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The concept of blood thromboplastin has been accepted by most workers in the clotting field since publication by Biggs and associates (1) of their classical studies on initial stages of blood coagulation. Bergsagel and Hougie (2) have shown that an activity corresponding to that of whole blood thromboplastin can be sedimented on platelets incubated in serum and recalcified plasma reagent, and they designated this activity "product 2." Similar activity has been sedimented on crude cephalin by Streuli(3); the present study deals with properties of such a thromboplastic preparation.

Materials and methods. Thromboplastin generation reagents were prepared as previously described(4), except that dilutions of all reagents were made with isotonic saline brought to pH 7.3 with imidazol buffer. Plasma reagent of human origin was diluted to 20% and bovine to 10%. The basic steps in preparation of blood thromboplastin were adapted from the method of Bergsagel and Hougie(2) as follows: one volume each of plasma reagent, serum reagent, and 0.025 M $CaCl_2$ were mixed and allowed to incubate at 37°C for 15 minutes. The clot which invariably formed was carefully removed, and an additional volume of cephalin reagent was added. Another 5 minutes incubation at 37°C gave a thromboplastin reagent which clotted recalcified human substrate plasma in 9-11 seconds. This was immediately centrifuged for 30 minutes and about 20,000 rpm at 4°C in an International PR-2 Refrigerated Centrifuge equipped with multi-speed attachment. The supernatant was discarded, and the sediment was resuspended in buffered isotonic saline containing 0.025 M $CaCl_2$. The mixture was again centrifuged as before, and the procedure repeated to give 2 washings. The last supernatant had no demonstrable thromboplastic activity. The final sediment was suspended in a volume of buffered calcium-saline equal to ¼ the volume of original generating mixture. By subsequent dilution this reagent was adjusted so that it clotted substrate human plasma in about 12 seconds. It was found that "cephalin" preparations from human brain or platelets(5) gave identical results. Plasma deficient in factor V was prepared by aging oxalated human plasma at 37°C for 3 days; plasma deficient in Stuart factor was kindly supplied by Dr. J. B. Graham from University of North Carolina.

Results. Blood thromboplastin obtained in the sediment was highly active, and had clotting properties described by others in whole thromboplastin generation mixtures(6). The reagent clotted normal, factor V-deficient, and Stuart factor-deficient plasmas equally well thereby demonstrating properties different from those of tissue thromboplastin (Table I). Blood thromboplastin preparation failed to clot 0.5% concentrations of Cohn's fraction I (Cutter) or substrate plasma with 0.1 cc of 0.38% sodium citrate substituted for calcium reagent, indicating absence of demonstrable thrombin activity.

TABLE I. Clotting Times in Seconds of Substrate Plasmas.

	Thromboplastin	
	Tissue	Blood
Normal	12	12
Factor V—deficient	37	13
Stuart factor—deficient	59	13

* This study was supported by USPHS Grant of Nat. Heart Inst.

TABLE II. Properties of Blood Thromboplastin.

Thromboplastin preparation	Human plasma substrate clotting time (sec.)
Fresh*	12
Incubated 1 hr at 37°C*	12
" 5 min. at 56°C*	28
" 10 min. at 56°C*	180
" 18 hr at 4°C*	20
" 18 hr at 4°C†	13
" 18 hr at 37°C†	60
Stored at 20°C for 24 hr*	23
Idem 3 wk*	56
Incubated 30 min. with equal vol of buffered saline*	17
Incubated 30 min. with equal vol of human serum*	40
Resuspended and washed sediment recovered from thromboplastin-serum incubation*	41

* Human plasma reagent.
† Bovine plasma reagent.

Some properties of blood thromboplastin are given in Table II. Storage stability was considerably greater than activity developed in the usual thromboplastin generation mixtures where there is rapid deterioration at 37°C(7). This increased storage stability probably results from removal of thromboplastin inactivator(s) in the washing process. Addition of citrated serum to the thromboplastin preparation resulted in rapid inactivation; and activity was not restored by resedimentation and washing twice as described above. Further increased storage stability was obtained when bovine instead of human plasma reagent was used. Activity was rapidly destroyed at 56°C and disappeared in the frozen state.

When the cephalin reagent was incubated with either plasma reagent or serum reagent in presence of calcium ion, the resulting washed sediment was unable to act as a combined reagent when used in a thromboplastin generation test, as shown in Table III. Thus, there is no evidence that the cephalin reagent was capable of adsorbing separate clotting factors in nonspecific fashion. Moreover, if the washing process was accomplished in calcium-free buffered saline, a preparation of poor activity was obtained. Activity was not restored by subsequent addition of Ca^{++}.

An experiment was performed in which plasma and serum reagents were used without dilution, and calcium and cephalin reagents were used in 10-fold concentration. Thromboplastin formation was performed as above, except that final thromboplastic mixture was kept incubating in 37°C water bath until all activity was gone, as measured by inability to accelerate clotting time of recalcified substrate plasma. This incubation took about 45 minutes. The mixture was then centrifuged and the sediment washed as described above, after which the sediment was suspended in a volume of buffered saline equal to that of starting volume of added cephalin reagent. A second mixture was prepared in identical manner except that calcium reagent was omitted: this preparation failed to clot, and no thromboplastic activity developed. The final suspensions of both preparations were used as cephalin reagent in the standard thromboplastin generation test, comparing their activity by serial dilution with an aliquot of the cephalin reagent originally used which had concurrently incubated at 37°C. Sediments resuspended from both mixtures showed identical activity, which was about half that of the free cephalin reagent. The loss probably represented incomplete sedimentation of cephalin, and no change could be attributed to inactivation by clotting. These findings are of interest in view of the recent report by Alexander *et al.*(8) that "serum platelets" poorly support thromboplastin generation. It is also evident that the sedimented material is phosphatide-containing.

Discussion. The thromboplastic material

TABLE III. Properties of Variously Prepared Thromboplastin Sediments.

Thromboplastin preparation	Human plasma substrate clotting time (sec.)
Plasma reagent, cephalin, Ca^{++}—Preincubated, washed, used with serum reagent in TGT	30*
Serum reagent, cephalin, Ca^{++}—Preincubated, washed, used with plasma reagent in TGT	28*
Blood thromboplastin washed with Ca^{++}	12
Blood thromboplastin washed without Ca^{++}	32
Idem, incubated 10 min. with added Ca^{++}	33

* After 6 min. incubation of generating mixture.

reported here appears identical in its properties to "product 2" of Bergsagel and Hougie (2). Our studies and those of Streuli(3) indicate that the only necessary component provided by platelets is their phosphatide. Since the thromboplastic material is probably a phosphatide-protein complex, and because of its sedimentation properties, it has characteristics resembling those of tissue thromboplastin prepared by Chargaff(9). It differs from the latter in its ability to convert prothrombin to thrombin in the absence of factor V or Stuart factor, and in its dissociation in the absence of calcium ion.

Our studies support the concept of blood thromboplastin formation as a chain reaction, since there did not appear to be haphazard complexing of clotting components to the phosphatide emulsion. This was demonstrated by failure of new activity to form when the cephalin reagent was incubated with either plasma or serum reagents separately. The data do not contribute to the question as to which clotting factors are complexed or whether any act in enzymatic manner. However, the present technics may provide a means by which such problems may be more effectively handled. As purified clotting factors become available, it would be of considerable interest to determine which are sedimented with appropriate clotting phosphatide.

Summary. A reagent with activity resembling that of blood thromboplastin was sedimented on crude cephalin. Activity was lost if washing of sediment was undertaken in absence of calcium ion. The thromboplastic reagent normally clotted plasmas deficient in Stuart factor or factor V; activity was irreversibly destroyed following incubation with serum. Certain properties similar to those of tissue thromboplastin are indicated.

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Action of Rabies Vaccine Derived from Embryonated Duck Eggs Against Street Virus. (25101)

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Two alternatives appeared possible in attempts toward decreasing the chances of encephalomyelitis following use of rabies vaccine derived from rabbit brain or other species of brain. These are: (1) chemical or physical treatment of the vaccine to remove as far as possible factors contributing to this type of side reaction, and (2) preparation of vaccine from virus infected tissue initially devoid of such factors. We experimented mainly along the latter line since some early results indicated fixed rabies virus could be grown reasonably well in embryonated duck eggs(1). Such duck embryo (DE) killed rabies vaccine suffices as a useful agent for treatment of persons bitten by animals or otherwise exposed to rabies street virus, and degree of antibody response and minimal extent of side reactions in such human patients have been reported(2). Although DE rabies vaccine fulfills requirements of potency and safety for human usage, we have thus far reported only

on its action against fixed virus. We have unpublished evidence concerning 90 dogs immunized with the vaccine and challenged intramuscularly at different times with street virus in experiments designed for veterinary use, and the results are good for the purpose intended. We here present results of further successful immunization experiments in which we used street virus challenge by intracerebral route according to Semple's(3) original monograph now nearly forgotten. His chapter V is particularly informative to this day. We followed very closely his original technic in tests of phenolyzed or "Semple vaccine."

Materials and methods. Five to 6 lb. normal rabbits were used. DE rabies vaccine of a lot exactly one year of age (in ice box storage) was used. This lot had passed all pertinent tests including potency maintenance after 30 days heating at 37°C. Street virus, as a first mouse passage from a fatal human case, was supplied by Dr. Edwin H. Lennette of Calif. State Dept. of Health Laboratories. Following their de-coding several left-over sera obtained from persons treated with DE and Semple rabies vaccines respectively were on hand following an antibody study of coded serum samples supplied by Dr. Morris Greenberg of N. Y. City Dept. of Health. These sera were used in serum + virus neutralization tests in standard white mice obtained from local breeders. The CVS strain of fixed virus was obtained from our laboratories, while Phillips and Park strains of fixed virus were obtained from our biological production laboratories. *Immunization with vaccine and challenge with street virus.* In following Semple's original procedure, we gave 8 rabbits each a subcutaneous dose of 1.5 ml of diluted 2% vaccine daily for 24 days. DE vaccine is 10% virus tissue when normally rehydrated, therefore it was diluted 1:5 further before use in rabbits. A week after last dose of vaccine, a small bleeding was taken from the rabbits and the resultant sera subjected to standard serum + virus neutralization test in mice. Then the rabbits, divided into 4 pairs, were injected intracerebrally with 0.25 ml street virus diluted 1:200, 1:400, 1:800, and 1:1600 respectively. A pair of normal control rabbits received each of these 4 dilutions of virus, also 2 additional pairs of normal controls received 2 higher dilutions of street virus, namely 1:3200 and 1:6400. *Serum + virus neutralization tests.* Sera of immunized rabbits and human patients' sera were tested for virus neutralizing antibody by standard methods using doubling dilutions of inactivated serum and virus so diluted that mice injected with the mixtures received 100 LD_{50} of virus/mouse. Groups of 6 mice were used/serum dilution, and 4 additional groups of 10 mice each were given amounts of virus computed to be 100; 10; 1.0; and 0.1 LD_{50} of live virus as controls. Rabbit sera were tested against CVS virus alone, while human sera were tested against CVS, Phillips, and Park fixed viruses, and also against street virus. Titers were computed by the method of Reed and Muench(4).

Results of street virus challenge. Table I shows results of successful immunization of rabbits with DE rabies vaccine in terms of resistance to intracerebral challenge with street virus. Semple found subdural inoculation of living (street) rabies virus a very severe test of immunity. He used 4 monkeys, 2 dogs, and 2 rabbits immunized with freshly made phenolyzed vaccine. All 4 monkeys, one of 2 dogs, and one of 2 rabbits survived subdural challenge. Rabbits immunized with DE vaccine resist several fatal doses, (up to about 32) of street virus, by more acute intracerebral challenge (Table I). It is of added interest that this vaccine was 1 year old when used. This action parallels that of freshly prepared Semple vaccine, and total dose of vaccine used/rabbit was the same as that used by Semple. It may be mentioned that DE vaccine is first DE passage fixed virus.

Results of neutralization tests. Table II shows results of virus neutralization tests of human sera following treatment with DE and Semple vaccines respectively. In addition to customary use of CVS fixed virus, we used 2 other strains of fixed virus, and also street virus. It appears there is little difference in the heterologous virus neutralizing action of serum following use of DE or Semple vaccine. The only differences noted are the strain to strain differences which both sera show simultaneously.

TABLE I. Rabbits Immunized with DE Rabies Vaccine and Challenged Intracerebrally with Street Virus.

Intracer. challenge dose of 0.25 ml of street virus diluted:	Rabbits immunized with DE rabies vaccine: Antibody titer	Result	Normal rabbits: Result	
1:200	85	Rabies, day 17	Rabies, day	15
	48	S	*Idem*	15
1:400	25	S	"	17
	35	S	"	15
1:800	14	Rabies, day 19	"	15
	26	S	"	15
1:1600	6	S	"	12
	27	S	"	13
1:3200	Not done		"	15
			"	15
1:6400	*Idem*		"	15
			S	

Street virus used was mouse brain passage from a fatal human case. Antibody titers are reciprocals of dilutions of serum computed to save half the mice from 100 LD_{50} of CVS virus/mouse.

S = surviving and normal at 30 days.

Discussion. Our DE vaccine had a "potency" (times better than N.I.H. standard vaccine No. 159A in mouse tests) of 1.27 when first made. Potency of a sample after heating 30 days at 37°C was 1.54, which indicates no decrease had taken place, and these figures are representative of the product (2). Degree of activity of this vaccine in rabbits is sufficient to cause these animals to resist from one to several fatal doses of street virus injected intracerebrally. This showing in light of Semple's results and those of many subsequent investigators using intracerebral street virus challenge is proof of direct capacity as an antirabies prophylactic. Human sera following use of DE and Semple vaccines respectively neutralize street virus as well as other fixed viruses in mouse tests. Titers of both kinds of sera, especially against street virus, are lower than when CVS fixed virus is used, however this would be anticipated by nearly all workers with these viruses. Such differences appear to be due to differences in sensitivity of virus to antibody.

TABLE II. Antibody Titers of Human Sera Measured against Various Rabies Viruses.

Serum following use of DE and Semple vaccine		Fixed virus strains: CVS	Phillips	Park	Street virus
(DE)	1-31-59	1010			128
"	1-14	1350	730	560	
"	1-29	1600	790	670	
(Semple)	3-26	1650		512	142
"	1-26	2450	850		

The above sera were of sufficient volume to allow only the number of tests shown. Antibody titers are expressed as in Table I.

Summary. Treatment of rabbits with DE rabies vaccine causes them to withstand otherwise fatal doses of street virus administered intracerebrally. These results with 1 year old DE vaccine parallel Semple's original experience with freshy prepared phenolyzed vaccine. Furthermore, human serum following treatment with these vaccines neutralizes different fixed viruses and also street virus as tested in white mice.

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Metabolism of Folic Acid in Folic Acid and Biotin Deficient Rat. (25102)

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In the course of a vitamin balance study with germ-free rats fed purified diets, Luckey *et al.*(1) observed that biotin administration to a rat reared on diet free of biotin and folic acid (pteroylglutamic acid, PGA) caused a 50-fold increase in PGA excretion over that

ingested each day. The observation, although limited to one rat, was interpreted as suggestive of tissue synthesis of PGA and its dependence on biotin. On the other hand, Halevy and Guggenheim(2) reported a study of PGA metabolism in certain vitamin deficiencies in the rat, that dietary biotin had no effect on hepatic levels of PGA and citrovorum factor (N^5-formyl tetrahydroPGA, CF) nor on conversion *in vivo* of PGA to CF. The present work was undertaken to study conditions under which biotin may influence PGA synthesis in the PGA-deficient rat.

Materials and methods. Young rats (Wistar Strain), of 40 g initial average weight were kept individually in raised-bottom wire-mesh cages on a purified diet deficient in biotin and PGA. The diet consisted of (in g/100 g): dried raw egg white(3) 10, hot alcohol-extracted casein 8, starch 60, sucrose (vitaminized) 10, salt mixture (U.S.P. No. IV) 4, sesame oil 4, shark liver oil 2 and succinyl sulphathiazole 2. Vitamin supplements, added through sucrose, provided (mg/kg diet): thiamine hydrochloride 4, riboflavin 5, niacin 10, calcium pantothenate 20, pyridoxine hydrochloride 5, menadione 3, inositol 500 and choline chloride 500. Sesame oil was fortified to give 10 mg α-tocopherol/kg of diet. A comparison group of PGA-deficient animals received a similar diet with devitaminized casein replacing egg white and with biotin included in the vitamin supplement at a level of 1 mg/kg diet. A group, serving as control, received supplements of biotin (1 mg/kg) and PGA (3 mg/kg) in the latter (18% casein) diet. After 8 weeks feeding, half of doubly deficient animals were each administered 100 μg of biotin intraperitoneally and placed on PGA-deficient casein diet for one week. Urine and feces were collected daily according to usual procedures during this (ninth) week for animals of the 3 deficient groups and food intake data were also recorded. All animals were sacrificed at end of 9 weeks under light ether anesthesia. Blood was obtained from portal vein and collected in citrated vials. Liver, spleen and kidney were excised with usual precautions and assayed for PGA and CF activities. The samples were homogenized in 10 volumes of ice-cold distilled water, aliquots taken in 0.1 M phosphate buffer, pH 7.4, and autolysed at 37°C for 18 hours under toluene. They were then steamed and centrifuged. PGA activity in supernatants was determined using modified medium of Mitbander and Sreenivasan(4) with synthetic PGA (Lederle) as standard and *Streptococcus faecalis* R as test organism. For CF activity determinations, the medium of Sauberlich and Baumann(5) was employed with Leucovorin (Lederle) as standard and *Leuconostoc citrovorum* (*Pediococcus cerevisiae*) as test organism. Erythrocyte and leucocyte count in blood samples were arrived at by standard hematological procedures and hemoglobin determined by the acid-hematin method. All results reported are averages of independent determinations from at least 4 animals in each group. Aliquots from urine and homogenized feces, collected for 7-day period, were autoclaved at 10 lb for 10 minutes after addition of ascorbic acid (10 mg/ml) which served to stabilize the labile reduced folate derivatives known to be excreted(6,7). Samples were then centrifuged and assayed for PGA. Amounts of PGA ingested were determined from data for food intake for each group and PGA analyses of diets fed. The results reported are daily averages determined from pooled samples collected during the week.

Results. Animals in the biotin and PGA-deficient group increased in weight from 40 to 140 g (averages) during 8 weeks, while PGA-deficient rats and the control group receiving all vitamins, had increased, respectively, to 170 g and 240 g (averages). Characteristic symptoms of biotin deficiency, such as alopecia and spectacle-eyes were also manifest in the former group. In ninth week of growth, deficient animals receiving biotin had increased in weight by an average of 14 g as compared to 2 g for biotin and PGA-deficient animals, 8 g for PGA-deficient animals and 12 g for control animals.

Data on blood and liver constituents are summarized in Table I. In simple PGA deficiency, marked reductions in liver and blood PGA and CF were accompanied with definite reductions in blood hemoglobin content (P

<0.001) and in leucocyte count ($P<0.05$). A superimposed deficiency of biotin was reflected in further reductions in liver levels of PGA ($P<0.001$) and CF ($P<0.02$), blood hemoglobin concentration ($P<0.005$) and, additionally, of blood erythrocyte count ($P<0.01$). However, there were no significant changes in blood levels of PGA ($P>0.1$) and CF ($P>0.1$). Administration of a single dose of biotin to doubly deficient animals caused in one week almost complete restoration of liver PGA ($P>0.1$) and CF ($P>0.5$) and blood hemoglobin ($P>0.1$) levels to those in the PGA deficient animals but the blood erythrocyte count was still maintained at a significantly lower level ($P<0.01$).

Observations on intake of dietary PGA and urinary and fecal excretions of the vitamin by deficient animals are recorded in Table II. The diet containing raw egg white carried more vitamin than the 18% casein diet and would account for the differences in PGA intake between groups, in spite of small differences in food intake. However, fecal excretion of PGA in biotin-fed groups was higher than in biotin-deficient animals and in fact exceeded amounts ingested. No differences were observed in urinary excretion of the vitamin which was low in all groups. The latter observation is more or less similar to blood levels of vitamin in these animals.

In a further experiment, weanling rats, reared on the PGA- and biotin-deficient diet for 8 weeks, were divided into 3 groups of 4 animals each. Each animal in one group was administered intraperitoneally 20 μg biotin daily while a similar amount of vitamin was fed orally by a catheter tube to animals of a second group. All 3 groups were continued on basal diet for a further week during which urine and feces collections were made as before. Animals were killed at end of 9 weeks for determinations in liver, spleen and kidney of total PGA. Table III shows that, while urinary excretion of PGA remained unaffected, tissue stored and, more markedly, fecal excretion of PGA increased on biotin administration. The extent of this stimulation was not significantly influenced by mode of administration.

Discussion. It appears that. while there

TABLE I. Effects of Biotin and Folic Acid (PGA) on Blood Picture and on PGA and Leucovorin (CF) Concentrations in Liver and Blood. Results represent mean values for at least 4 animals from each group ± stand. error of mean.

Group*	Erythrocytes/mm³ (× million)	Leucocytes/mm³ (× 1000)	Hemoglobin, g/100 ml	Liver		Blood	
				PGA	CF	PGA	CF
				μg/g		μg/100 ml	
1. Biotin and PGA-deficient	6.13 ±.27	5.86 ±.45	11.88 ±.34	.563 ±.016	.156 ±.011	.291 ±.017	.077 ±.016
2. *Idem*, administered biotin	6.08 ±.32	6.90 ±.36	12.63 ±.66	.660 ±.017	.201 ±.018	.330 ±.019	.115 ±.015
3. PGA-deficient	8.03 ±.45	6.40 ±.34	13.68 ±.10	.695 ±.015	.217 ±.017	.337 ±.020	.117 ±.015
4. Biotin and PGA-fed	8.13 ±.55	7.65 ±.32	15.21 ±.23	4.730 ±.320	3.210 ±.430	2.760 ±.130	2.130 ±.210

* Young rats were fed diets deficient in PGA (group 3) or PGA and biotin (groups 1 and 2) for 8 wk when half the doubly deficient animals (group 2) were given each a single dose, intraper., of 100 μg biotin. All animals were continued on their respective diets for another week when they were killed for determinations. A comparison group (Group 4) received both PGA and biotin.

TABLE II. Effect of Biotin on Folic Acid (PGA) Excretion in PGA-Deficient Rat. Results represent mean values for at least 4 animals from each group ± S.E.

Group*	Food intake during 7 days, g	PGA intake	PGA excretion — Urine	PGA excretion — Feces
		mμg/rat/day		
1. Biotin and PGA-deficient	98 ± 5	472 ± 20	50 ± 12	267 ± 12
2. *Idem*, administered biotin	95 ± 5	423 ± 27	53 ± 6	507 ± 13
3. PGA-deficient	93 ± 9	415 ± 40	46 ± 6	487 ± 17

* For details of grouping, see Table I. Urine and feces were collected for analysis during all of 9th wk of experiment.

may be differences in rates of recovery from biotin deficiency, administered biotin increased availability of PGA to the organism. These observations, however, differ from those of Halevy and Guggenheim(2) who reported that biotin causes no significant increase in hepatic PGA or CF concentrations as well as in *in vivo* conversion of PGA to CF in the tissue.

The effect of biotin on fecal excretion of PGA is more marked than on liver levels of the vitamin; together with low values for urinary excretion and blood levels of PGA, these observations suggest that biosynthesis of PGA occurs in the intestinal tract, rather than in body tissues, through some, as yet unclear, influence of biotin. Such an effect apparently prevails even in succinyl sulphathiazole-added diets.

In a study on synthesis of PGA-active compounds by growing cells of *Lactobacillus arabinosus* 17-5 in presence of p-aminobenzoic acid, Mitbander and Sreenivasan(4) reported that biotin and xanthine, among various metabolites tested, showed marked stimulation of PGA biosynthesis. Use of Tween 80 (polyoxyethylene sorbitan monoleate) in the medium also stimulated PGA synthesis. The effects due to biotin and Tween 80 were not, however, additive. It was concluded that stimulation afforded by these compounds could be through increased surface activity and consequent cell permeability to precursors needed for PGA biosynthesis. Such an effect due to biotin may explain the observed stimulation of PGA synthesis by intestinal microorganisms.

Since the animals were deficient in PGA, it is unlikely that the large amounts of PGA excreted in feces are of tissue origin. It is more probable that biotin, whether administered orally or intraperitoneally, becomes available to intestinal microflora, thence causing increased synthesis of PGA, part of which becomes available to the host animals.

Our experiments neither demonstrate nor exclude the possibility of tissue synthesis of PGA, but point out that biotin administration to PGA-deficient rats may augment intestinal synthesis of PGA, and, as a consequence, improve tissue levels of the vitamin.

Summary. A deficiency of biotin, superimposed over a simple PGA deficiency in rats resulted in further reductions in liver levels of PGA and CF, blood hemoglobin and, additionally, blood erythrocyte count. Administration of biotin to doubly-deficient animals caused almost complete restoration of liver levels of PGA and CF and of blood hemoglobin; under these conditions there was

TABLE III. Effect of Biotin on Tissue Retention of Folic Acid (PGA) and Its Excretion. Results represent mean values for at least 4 animals from each group ± S.E.

Group*	PGA excretion — Urine	PGA excretion — Feces	Tissue content of PGA — Liver	Spleen	Kidney
	mμg/rat/day		mμg/g		
1. Biotin-deficient	42 ± 6	222 ± 22	550 ± 20	6 ± 2	310 ± 30
2. " -ingested	43 ± 3	468 ± 12	620 ± 13	16 ± 5	493 ± 15
3. " -injected	41 ± 5	420 ± 10	610 ± 21	12 ± 4	400 ± 14

* Weanling rats were reared on PGA and biotin-deficient diet 8 wk after which they were divided into groups receiving biotin (20 μg/rat/day) either orally (group 2) or intraper. (group 3). Group 1 served as control. Urine and feces collections were for 9th wk after which animals were killed for tissue analysis of total PGA.

marked excretion of PGA in feces, but not in urine, the amount excreted exceeding by far the tissue rise in level of vitamin. It is suggested that biotin administration may influence synthesis of PGA by intestinal microflora and, as a consequence, improve tissue levels of the vitamin.

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Formation of Creatine from Guanidinoacetate in Pancreas.* (25103)

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Several investigators established that 2 enzymes are involved in biosynthesis of creatine (1). Arginine + glycine ⇄ guanidinoacetate + ornithine. Guanidinoacetate + S-adenosylmethionine → creatine + S-adenosylhomocysteine. The first enzyme, arginine-glycine transamidinase, has long been known to occur in mammalian kidney(2), while the second enzyme, guanidinoacetate methylferase, has been found in liver(3,4). Previously it has been assumed that under physiological conditions these 2 synthetic reactions are carried out in successive steps in different organs of the mammal. Guanidinoacetate was believed to be synthesized in the kidney from arginine plus glycine and then transported *via* the blood stream to the liver, where methylation to creatine took place. Recently, however, another mammalian organ has been implicated in creatine biosynthesis, with the discovery that pancreas contains a high concentration of arginine-glycine transamidinase (5). Since guanidinoacetate can be synthesized in pancreas, it became of interest to determine whether methylation of guanidinoacetate to form creatine might also occur in this organ. The purpose of this paper is to present evidence for occurrence of guanidinoacetate methylferase activity in mammalian pancreas.

Methods. The enzyme preparations were dialyzed beef or dog pancreas fractions which precipitate between 30% and 60% saturation with ammonium sulfate, prepared as described elsewhere(5). All incubation mixtures contained pancreas enzyme preparation, 24 mg; tris (hydroxymethyl) aminomethane buffer, pH 7.6, 100 μmoles; other additives as noted in individual experiments; final volume, 1 ml. Tubes were incubated 2 hr at 37°C; the reactions were terminated by adding 1 ml water and heating 3 min at 100°C. In the time-course experiment with guanidinoacetate-2-C^{14}, 5 μliters were removed from each reaction mixture at times indicated and spotted on paper chromatograms. Non-labelled guanidinoacetate and creatine were added to each spot as carriers. After development of the paper chromatograms with buffered phenol, the separated compounds were located with alkaline ferricyanide-nitroprusside spray. Areas of the paper containing creatine and guanidinoacetate were cut out, eluted, dried, and counted. In experiment with glycine-2-C^{14}, non-labelled guanidinoacetate and creatine were added as carriers to the reaction mixtures immediately prior to termination of incubation. Guani-

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† Senior Research Fellow of P.H.S.

TABLE I. Synthesis of Creatine from Various Precursors in Beef Pancreas.

Precursors		μmoles creatine formed
(a) Arginine	.47 μmoles	.21
+ Glycine	1.3 "	
+ S-adenosylmethionine	1.7 "	
(b) Guanidinoacetate	.51 "	.23
+ S-adenosylmethionine	1.7 "	

dinoacetate and creatine were separated from the deproteinized solutions by paper chromatography, and were eluted and counted as described above.

Results. Preliminary experiments were carried out to determine if guanidinoacetate methylferase is present in mammalian pancrease in high enough concentration to be physiologically significant. One way to determine this is to compare activity of pancreas with that of a tissue known to have high guanidinoacetate methylferase activity, *e.g.*, guinea pig liver(3). The tissues to be compared were homogenized, centrifuged, dialyzed, and assayed; the creatine formed was determined by a diacetyl method(6). The relative enzyme activities were as follows: guinea pig liver, 100; dog pancreas, 73; dog liver, 26. These results encouraged the more extensive experiments described below.

The experiment summarized in Table I demonstrates that creatine can be synthesized by beef pancreas preparation from either guanidinoacetate or arginine plus glycine, with S-adenosylmethionine‡ as the methyl group donor. The versatility of pancreas with respect to creatine precursors is clearly apparent in this experiment. In contrast to pancreas, mammalian liver cannot synthesize creatine from arginine, glycine, and S-adenosylmethionine, since liver lacks the enzyme arginine-glycine transamidinase. Although the diacetyl method was employed here for assay of creatine(6), the assay involving conversion to creatinine, adsorption and elution from Lloyd's reagent and color development with alkaline picrate(7), gave similar results in independent experiments.

‡ S-Adenosylmethionine bromide (80% pure) was purchased from California Corp. for Biochemical Research.

Unfortunately, both of the colorimetric assays for creatine mentioned above are relatively non-specific(4). Consequently to establish the presence of guanidinoacetate methylferase in pancreas beyond a reasonable doubt, additional experiments were performed with radioactive substrates. In these experiments reactants and products were isolated by paper chromatography, eluted, and counted (8). Fig. 1 shows time-course of conversion of guanidinoacetate-2-C^{14} to creatine, as catalyzed by beef pancreas preparation, in presence and absence of the methyl group donor, S-adenosylmethionine. No creatine was formed in the absence of added methyl donor.

Table II summarizes an experiment in which glycine-2-C^{14} was employed as a creatine precursor, with arginine as the formamidine group donor and S-adenosylmethionine as the methyl donor. It can be seen that glycine was not incorporated into creatine in significant amounts in the absence of added methyl donor; however, guanidinoacetate was formed from glycine and arginine. On the other hand, when S-adenosylmethionine was present, glycine was converted all the way to creatine, with a lesser amount accumulating in the intermediate guanidinoacetate. This conversion of glycine to guanidinoacetate and to creatine was completely inhibited by ornithine, a powerful inhibitor of arginine-glycine transamidinase(5). Since separate experi-

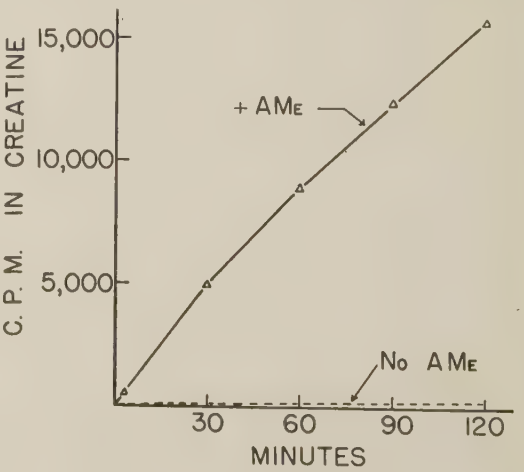

FIG. 1. Time-course of conversion of guanidinoacetate-2-C^{14} to creatine in beef pancreas. Guanidinoacetate, 0.25 μmole, 22,000 cpm; S-adenosylmethionine (AMe), 3.3 μmoles.

TABLE II. Incorporation of Glycine-2-C^{14} into Guanidinoacetate and Creatine in Beef Pancreas.

Compounds added		cpm in Guanidinoacetate	cpm in Creatine
		(× 100)	(× 100)
(a) Glycine-2-C^{14}, 37,000 cpm	.27 μmoles	239	2
+ Arginine	.95 "		
(b) *Idem* + S-adenosylmethionine	3.3 "	86	157
(c) " + S-adenosylmethionine	3.3 "	2	5
+ Ornithine	18 "		

ments had established that ornithine does not inhibit guanidinoacetate methylferase activity, these results provide further evidence that guanidinoacetate is an intermediate in the incorporation of glycine into creatine.

Discussion. It is now apparent that mammalian pancreas contains physiologically significant concentrations of both enzymes involved in the biosynthesis of creatine from arginine, glycine, and S-adenosylmethionine. Although it would appear that biosynthesis of creatine might be more efficiently carried out *in toto* in one organ (pancreas) rather than in successive steps in different organs, further work must be done before the relative importance of the 2 pathways can be assessed.

In either event, an important physiological role for pancreas in the biosynthesis of creatine precursor, guanidinoacetate, seems assured(5). Kidney, the other organ capable of participating in guanidinoacetate synthesis, may play only a supporting role in this respect, since its arginine-glycine transamidinase concentration is lower, and its arginase activity is much higher than that of pancreas.

Summary. An enzyme preparation from mammalian pancreas catalyzes the synthesis of creatine from guanidinoacetate and S-adenosylmethionine. Inasmuch as pancreas, in contrast to liver, can also synthesize the creatine precursor, guanidinoacetate, from arginine plus glycine, it would appear that pancreas may play a unique role in biosynthesis of body creatine.

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Modification of Protein Catabolism in the Anuric Dog.* (25104)

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Numerous studies have been reported on urea accumulation and survival time in anuric animals under various conditions. There have been, in general, beneficial effects from carbohydrate feeding and harmful effects from fasting or protein feeding(1). These results are presumably due to modification of the protein catabolic rate in each case. The response to testosterone has been variable, depending on diet used and presence or absence of catabolic accelerators such as tissue necrosis or

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TABLE I. Results of Treatment with Hydrocortisone and Testosterone in Anuric Dogs.

	Survival time (hr)	Blood urea N (mg/100 ml)	Creatinine (mg/100 ml)	Phosphorus (mg/100 ml)
		48 hr post-op.		
Control	96 ± 17	150 ± 18	17.2 ± 7.7	9.1 ± 2.2
Hydrocortisone	*128 ± 33	*101 ± 37	* 7.5 ± 2.0	*5.6 ± 1.0
Testosterone	103 ± 22	142 ± 26	* 5.6 ± 1.0	*6.3 ± .8

* $p < .01$

infection(1). Under certain conditions, ACTH increases rate of urea accumulation in the nephrectomized rat while cortisone does not(2). Our experiments were performed to compare effects of hydrocortisone and testosterone on survival time, urea accumulation and other parameters in the anuric dog. The confounding effects of diet and hydration were excluded by experimental design. In anuric animals, serial concentrations of blood urea nitrogen were used to estimate rate of urea accumulation, and thus, protein catabolic rate(3). This technic depends on a relatively stable volume of total body water and absence of extra-renal loss of urea during period of observation.

Materials and methods. Mongrel dogs of both sexes living under ordinary kennel conditions had measurements made of their body weight, hematocrit, plasma concentration of urea, creatinine, phosphorus, sodium, potassium and CO_2 on 2 successive days. Standard laboratory methods were employed. CO_2 was measured as the "CO_2 combining power." Following the control period each dog was subjected to bilateral ureteral ligation under sodium pentothal anesthesia and assigned to one of 3 groups as described below. All dogs in all groups had daily post-operative measurements corresponding to preoperative measurements. No dog was allowed food or water post-operatively for duration of the animal's survival. *Group A.* 17 dogs served as controls and were given no drugs following surgery. *Group B.* 7 dogs were given hydrocortisone, 100 mg intravenously immediately following surgery and daily thereafter by same route. *Group C.* 8 dogs were given testosterone propionate, 50 mg intramuscularly immediately following surgery and daily thereafter.

Results. Preoperative measurements were normal in all respects. Post-operatively, there was a highly significant prolongation of survival time and a lower rate of urea accumulation in the hydrocortisone treated group. The 48-hour post-operative blood urea nitrogen concentration was used because it includes the largest number of surviving animals. The differences, however, persist at the same level of significance at 72 and 96 hours. Both hydrocortisone and testosterone treated groups showed a significantly lower serum creatinine and phosphorus concentration at 48 hours than did the controls. Means, standard deviations, and levels of significance are presented in Table I.

There were no significant differences among the groups with respect to decrement of body weight, decrement of plasma CO_2 concentration, or increment of plasma potassium concentration. The pooled means were respectively, 0.4 kg/day, 1.9 meq/l/day and 1.3 meq/l/day.

Discussion. The similarity of weight loss in the 3 groups suggests that changes in total body water were approximately equal. This observation, as well as lack of difference in hematocrits among the 3 groups rules out any major differences in total body water and plasma water. On this basis, the lower blood urea nitrogen concentrations in the hydrocortisone treated group may be assumed to represent a lower rate of protein catabolism. The action of hydrocortisone in prolonging survival time and lowering accumulation rates of urea, creatinine, and phosphorus is, therefore, in contrast to its effects in non-uremic animals. The association of a prolonged survival time with a lowered rate of urea accumulation in the hydrocortisone treated group is in accord with the widely held view that urea concentrations serve as an index of the presence of unknown, toxic protein catabolites.

The failure of testosterone to affect significantly survival times and urea accumulation may be related to duration of survival. Where-

as blood urea nitrogen concentrations in the hydrocortisone treated group rose in a linear fashion, those in the testosterone treated group showed a marked tendency to flatten out in the last day or 2 of the animal's life.

Summary. Hydrocortisone, given to anuric dogs, prolonged survival time with lower rates of accumulation for urea, creatinine and phosphorus in the serum. Testosterone in a second group of animals failed to influence survival time or urea accumulation.

Technical assistance of Gerald F. Rosenthal is gratefully acknowledged.

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Elevation of γ-Aminobutyric Acid in Rat Brain with Hydroxylamine.* (25105)

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γ-Aminobutyric acid (γABA)† has been found in all areas of mammalian central nervous system which have been subjected to analysis. A number of hypotheses have been advanced to account for a variety of physiological effects attributed to γABA(1-11), but none has received unequivocal experimental proof. The levels of γABA in brains of adult animals are relatively constant and show little variation from one animal to another in inbred strains of mice(12). Cerebral levels of γABA have been lowered in rats and mice by administration of Vit. B_6 antimetabolites and carbonyl trapping agents(10,13-15), but a sustained elevation of γABA in brains of normal animals has not been produced. Injection of massive doses of γABA into mice, cats, and rats produced no significant changes in brain levels of γABA(12,16) or electroencephalographic recordings(4). More than ½ of any trace dose of isotopically labeled γABA injected intracerebrally into mice disappeared within 2 minutes from the brain(11) and rapid labeling of other amino acids and tricarboxylic acid cycle intermediates occurred indicating that there is a rapid rate of metabolism of exogenously supplied γABA. Selection of NH_2OH for the study reported here was based upon observations which indicated that *in vitro* NH_2OH was a more effective inhibitor of the γABA-α-ketoglutaric acid transaminase than of glutamic acid decarboxylase (17-19) and might, therefore, cause an accumulation of γABA.

Methods. All experiments were performed with male rats of either Sprague-Dawley or Wistar strain. Treated animals were injected intraperitoneally with a solution containing 50 mg/ml NH_2OH (pH 7.0). The toxic effects observed after administration of NH_2OH are believed to result from methemoglobin formation(20) and involvement of central nervous system(21). To minimize the hematological effect some rats received an intraperitoneal injection containing 10 mg of MB in 0.5 ml(22). The dye was administered 10 to 20 minutes prior to treatment with NH_2OH. Control animals received no treatment. Intraperitoneal injections of physiological saline previously had no effect on levels of γABA in the brains of rats(10). One to 1½ hours after injection of NH_2OH the rats were killed by decapitation. Brain areas were removed

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† The following abbreviations have been used: γ-aminobutyric acid - γABA, hydroxylamine - NH_2OH, methylene blue - MB, triphosphorpyridine nucleotide - TPN.

immediately, weighed and dropped into separate vials containing 4 to 6 ml of ice cold 80% ethanol. Samples were homogenized in Tembroeck homogenizer and transferred quantitatively to 12 ml centrifuge tubes. Two to 4 ml of 75% ethanol were used to rinse homogenizer and pestle. Rinsings were added to homogenate. The homogenate was spun at 2000 g for 15 minutes and supernatant decanted. The precipitate was resuspended twice in 3 ml aliquots of 75% ethanol and spun. Extracts of each sample were pooled in 20 ml beaker and evaporated to dryness. One ml of distilled water was added to dried extract for every 100 mg of original fresh wt of tissue. The extract was resuspended and the suspension was spun in Spinco ultracentrifuge at 20,000 g for 30 minutes and aliquots of supernatant (0.2 to 0.25 ml) pipetted into numbered optical tubes. The supernatant was evaporated to dryness and stored in vacuum desiccator. The extract in optical tubes was resuspended in 0.1 ml of water and 0.3 ml of pyrophosphate buffer and analyzed by the enzymatic method described below. Quantitative recoveries of γABA added to original homogenate were achieved routinely. γABA was measured enzymatically by a new method. The bacterium *Pseudomonas fluorescens* EC, when grown on pyrrolidine, contains an enzyme system which converts γABA to succinate *via* transamination and oxidation coupled to TPN reduction(25). A quantitative assay for γABA has been developed by use of this enzyme system(24). The method was adapted to measurement of γABA in extracts of rat brain. Fraction 3, the first dialyzed acetone fraction of the enzyme system (25), was used for all determinations of γABA. The assay system consisted of the following: 0.1 ml of tissue extract equivalent to 20 or 25 mg of fresh brain tissue; 0.3 ml of 0.1 M pyrophosphate buffer, pH 8.3; 0.1 ml mercaptoethanol, 2 mg/ml in pyrophosphate buffer; 0.1 ml TPN, 8 mg/ml; 0.1 ml enzyme; 0.1 ml α-ketoglutaric acid neutralized with sodium hydroxide, 14.6 mg/ml. Final volume of assay system was 0.8 ml. The mercaptoethanol, TPN, enzyme and α-ketoglutarate were premixed and stored on ice. The reaction was started by addition of 0.4 ml of the mixture to 0.4 ml of buffer and sample contained in optical tube at room temperature. Optical density at 340 mμ was read immediately after stirring the reaction mixture. All spectrophotometric readings were made with Beckman Spectrophotometer model DU, equipped with tube adapter(26). The reaction proceeded at room temperature. Maximal readings at 340 mμ were reached 15 to 20 minutes after starting the reaction.‡ Maximal change in OD_{340} from zero time was proportional to amount of γABA in a sample. One μg of γABA in the system corresponded to ΔOD_{340} of 0.055. This represents close to an isomolar relationship between γABA in a sample and formation of TPNH. Samples containing no γABA showed no changes in OD_{340}.

Results. Male Sprague-Dawley rats, pretreated with MB and receiving NH_2OH at 100 mg/kg body wt., convulsed within 5 minutes after intraperitoneal injection of the drug. Convulsions, which in no instance lasted longer than 10 minutes, were accompanied by visible blanching of extremities. Fifteen minutes after injection, breathing still was rapid and labored, but returned to normal within 30 to 60 minutes. At time of decapitation all treated animals appeared slightly sedated but otherwise normal. Blood showed some discoloration because of presence of MB and methemoglobin. There were marked elevations of γABA in all 8 brain areas of treated rats by comparison with controls (Table I). The largest relative increase in content of γABA was noted in the cortex. Chromatographic verification of elevation of γABA in the cortex of treated animals is shown in Fig. 1. Increase in freely extractable γABA was by far the most notable alteration produced in the content of detectable ninhydrin-reactive constituents.

In view of possible effect of MB on γABA levels, an experiment was performed with 4 weight-matched groups of 3 male Wistar rats each. Rats in group 1 received no treatment; group 2 received an injection of MB only;

‡ Enzyme concentration was so adjusted that 0.1 ml of enzyme in incubation mixture of 0.8 ml produced maximal reading at OD_{340}, 15 to 20 minutes after initiating the reaction.

TABLE I. Effect of Hydroxylamine upon Brain Levels of γABA in Rats Pretreated with Methylene Blue.

Area	Control γABA (mg %)		Treated γABA (mg %)		Increase (%)
Olfactory lobe	40*	37–43†	61	52– 69	53
Diencephalon	56	51–61	79	64– 98	41
Superior colliculi	57	56–58	91	81–109	60
Inferior "	45	40–50	80	68–103	78
Cortex	26	24–27	48	43– 59	85
Cerebellum	32	31–34	44	41– 52	38
Pons	25	23–28	36	32– 42	44
Medulla	26	22–29	33	29– 39	27

* Avg † Range

Experiment was performed with Sprague Dawley rats 200 to 250 g in wt. All treated animals received one intraper. inj. of methylene blue (10 mg in 0.5 ml) and 20 min. later an intraper. inj. of NH_2OH (25 mg in 0.5 ml pH 7). Animals were decapitated between 60 and 75 min. after inj. of NH_2OH.

group 3 received relatively low dose of NH_2OH (10 mg); and group 4 received both MB and higher dose of NH_2OH (15 mg). The results are shown in Table II. MB alone tended to decrease γABA levels. Despite lower dosage of NH_2OH employed, the brains of rats in group 3 (Table II) showed marked elevation of γABA levels. The blood of these animals showed no visible discoloration.

Changes of cerebral γABA with time in cortex of rats treated with NH_2OH are shown in Fig. 2. The results suggest that at the time of convulsions (5 minutes after injection) γABA levels in brains of treated rats were not elevated. Raised levels of γABA in the cortex occurred concomitantly with lethargic behavior of these animals 1 to 2 hours after injection. The following report will discuss electrophysiological findings(27).

Discussion. It has been difficult to show that a causal relationship exists between decrease in cerebral levels of γABA, antivit. B_6 activity, and seizures. Convulsions and seizures induced by administration of Vit. B_6 antimetabolites and by convulsant hydrazides are accompanied by lowering of γABA levels (13,10,15). Furthermore, it has been reported that such convulsions can be prevented or stopped by administration of Vit. B_6 itself, by pretreatment with parenterally-adminis-

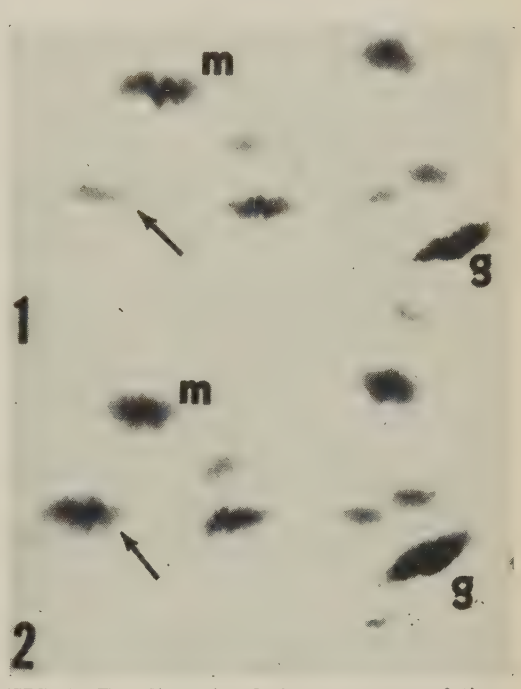

FIG. 1. Two-dimensional chromatograms of tissue extracts corresponding to 25 mg (wet wt) of cortex in untreated rats (1) and those treated with NH_2OH (2). Descending 2-dimensional chromatography was performed in water-saturated phenol and water-saturated lutidine. Amino acids were visualized by spraying with solution of 0.1% ninhydrin in butanol. The following symbols have been used to identify amino acids on chromatograms. Arrow points to γABA, g = glutamic acid, m = marker of α-aminobutyric acid.

TABLE II. Separate Effects of Methylene Blue and Hydroxylamine upon Brain Levels of γABA in Rats.

Group	Treatment		γABA levels in different areas of brain (mg %) Diencephalon	Colliculi	Olfactory lobes	Cortex	Cerebellum
1	Control		58	57	42	27	28
2	Methylene blue	10 mg	38	45	35	22	22
3	Hydroxylamine	10 "	71	71	55	47	40
4	Methylene blue + hydroxylamine	10 " 15 "	82	89	62	51	39

Each group consisted of 3 Wistar male rats 160 to 190 g in wt. Animals were decapitated 70 to 90 min. after last inj. and brain areas removed and extracted as described in text. Hydroxylamine inj. corresponded to dose of 50 to 65 mg/kg in Group 3, and 75 to 93 mg/kg in Group 4.

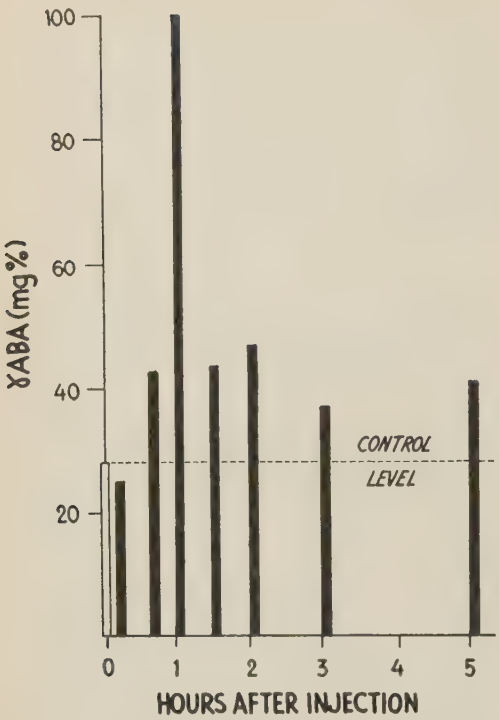

FIG. 2. Changes with time in levels of γABA after inj. of hydroxylamine. Each bar represents level of γABA in cortex of one Wistar male rat inj. with hydroxylamine and decapitated at different times after inj. The inj. dose corresponded to 10 mg/150 g body wt. Animals ranged in wt from 150 to 200 g.

tered γABA(14,28,29), or by topical application of γABA to specific areas of brain(30). Probably no appreciable amount of parenterally-administered γABA enters brain tissue unless the blood-brain barrier is destroyed(4) and the effects produced by topical application of γABA to exposed cortex are difficult to interpret(31).

The case for a causal relationship between increases or decreases in seizure susceptibility in specific brain areas with corresponding increases or decreases in γABA level could be strengthened if endogenously raised γABA levels would result in electrophysiologically observable effects which were opposite to those found with convulsant hydrazides or B_6 antimetabolites. A large number of drugs we tested failed to elevate γABA levels in whole brains of mice, rats, and rabbits(12). Experiments with NH_2OH are the first to show that increase in endogenous levels of γABA can be produced in normal animals by chemical means. If elevation of endogenous levels of γABA should decrease seizure susceptibility, then NH_2OH or one of its derivatives may find practical use in control of clinical seizures.

Although NH_2OH was tested because of its potent inhibitory action on γABA-α-ketoglutarate transaminase, it is by no means certain that this is the mechanism by which elevation is achieved *in vivo*. Besides combining with pyridoxal and keto acids, NH_2OH may also exert its effect through interaction with the DPN(32) of succinic semialdehyde dehydrogenase(33). Indirect effects of NH_2OH through action of its postulated metabolic products such as nitrite(34), nitrogen or ammonia(35), or through cerebral anoxia resulting from methemoglobinemia seem less probable, but cannot be completely excluded as contributing toward changes in levels of cerebral γABA. While evaluation of these possibilities is underway, the use of NH_2OH will provide a convenient tool for investigating the physiological effects which accompany elevated γABA levels in the central nervous system.

Summary. 1. Intraperitoneal injection of hydroxylamine resulted in elevation of levels of γ-aminobutyric acid in 8 brain areas of the rat. 2. These elevated levels of γ-aminobutyric acid were attained even after rats were pretreated with methylene blue to reduce methemoglobin formation. 3. The effect of a single injection of hydroxylamine persisted for at least 5 hours. 4. The potential value of this procedure for physiological study has been discussed.

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Anticonvulsant Properties of Hydroxylamine and Elevation of Cerebral γ-Aminobutyric Acid in Cats.* (25106)

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In the preceding paper(1) it was shown that elevation of brain levels of γ-aminobutyric acid (γABA) could be achieved in the rat by injection of hydroxylamine (NH_2OH). Since a correlation exists between decreases in brain γABA and increased seizure susceptibility(2,3 also see 1 for other pertinent references), it was of interest to determine whether a decrease in induced seizure activity would be found when brain levels of this substance are elevated. The present report is concerned with the effect of NH_2OH on electrically-induced seizure afterdischarges in acute experiments in cats.

Methods. The cats used were immobilized with gallamine triethiodide [Flaxedil®] and anesthetized locally with procaine at painful points. Preliminary surgical procedures, including a wide bilateral craniectomy, were carried out under ether anesthesia. The ether was blown off during 1½ hours of artificial ventilation. Two samples of cortex, one on

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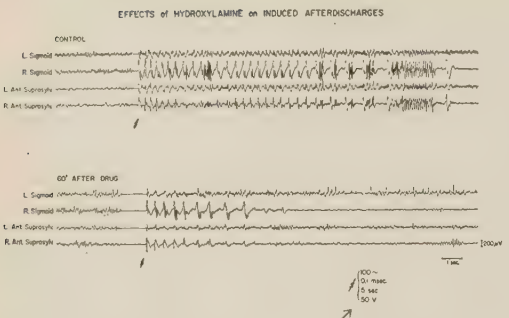

FIG. 1 (EEG records). At the S markings afterdischarges were induced by electrical stimulation near the R. sigmoid lead at parameters specified above. Recording was interrupted during stimulation. 10 mg/kg of NH_2OH were used.

each hemisphere but from nonhomologous loci, were then taken surgically as controls for γABA assay, hemostasis was completed, and multiple cortical electrodes were laid over exposed cortex for EEG recording. Two pairs of similar chlorided-silver ball electrodes (connected to Grass S4C stimulators and isolation units) were used for induction of afterdischarges. Fixed parameters for stimulation were: 100 c/s, 0.1 msec rectangular pulses, 5 sec. trains. Voltage was increased until consistent afterdischarge patterns were observed, with spread to at least one lead away from stimulated site. At least 3 control electrical seizures were obtained in each locus before administration of the test drug. Dilute neutralized solutions of NH_2OH or sodium nitrite in saline were given intravenously. Cortical EEG was monitored continuously thereafter. In each locus tested stimulations were repeated at 10 minute intervals at same fixed voltage as before drug administration for periods one to 4 hours. Two more cortical samples were taken for biochemical analysis at end of test period. Care was taken to obtain samples from cortical loci opposite to each control sample to minimize variations. All samples were weighed quickly and stored in 70% ethyl alcohol in the cold prior to assay for content of γABA.

Results. A brief (3-4 minutes) period of depression in electrocortical activity could be observed immediately after single doses of NH_2OH between 10-20 mg/kg were given. A gradual return to normal record took place in a few more minutes. This depression could be avoided by administering the same total dose in several injections spaced at 10-15 minute intervals. In some animals definite increases in seizure after discharges were observed within the initial 20 minute period after injection. After 30 minutes and up to 3 hours induced seizures were much shorter and spread to fewer leads than during control period and often were restricted to the lead next to the stimulating electrodes (Fig. 1 and Table I). In one cat it was necessary to raise the stimulating voltage considerably to elicit any seizures at all.

Levels of γABA in the brain samples are shown in Table II. Increases of 100% or more over control levels were obtained in 4 of the 5 NH_2OH-treated animals studied. The

TABLE I. Influence of Hydroxylamine and Sodium Nitrite on Electrically-Induced Seizure Afterdischarges in Cats.*

Drug	Dose, mg/kg	Locus of test	Pre-drug		Post-drug (60 min.)†	
			Mean seizure duration, sec.	Spread to channels, No.	Mean seizure duration, sec.	Spread to channels, No.
None		R. ant. suprasylv.	10	4	12	4
NH_2OH	20	R. sigmoid	18	5	4	2
		R. post. suprasylv.	12	2	5	1
"	10	L. sigmoid	10	2	5	1
		R. post. suprasylv.	8	3	3	1
"	20	L. middle suprasylv.	20	4	2	1
		R. ant. suprasylv.	7	4	0	0
$NaNO_2$	20	L. middle suprasylv.	12	4	28	4
		R. ant. suprasylv.	10	4	32	5

* Experimental parameters are described in text.

† Although results are shown for 60 min. period only, measurements were made in all instances for several hours at 10 min. intervals.

TABLE II. Influence of Hydroxylamine and Sodium Nitrite on Cerebral Levels of γ-Aminobutyric Acid in Cat.

Drug	Dose, mg/kg	Area	γ-Aminobutyric acid level Pre-drug, mg %	Post-drug, mg %	Time, min.
NH_2OH	10	Post. ectosylv.	16.9	24.9	40
		Sigmoid	17.2	25.1	
"	"	Post. ectosylv.	22.4	41.7	60
		Sigmoid	18.3	46.0	
"	"	Post. ectosylv.	19.3	42.2	70
		Sigmoid	19.8	49.1	
"	20	Middle ectosylv.	19.6	44.9	90
		Ant. suprasylv.	21.0	41.0	
"	"	Post. ectosylv.	16.5	38.8	105
		Sigmoid	18.7	39.0	
$NaNO_2$	60	Post. ectosylv.	18.4	19.7	30
		Sigmoid	19.1	23.0	
"	20	Post. ectosylv.	22.6	33.0	75
		Ant. suprasylv.	21.4	24.0	

fifth animal was exposed to the drug for less than one hour and showed an increase of approximately 50%.

Reduction of seizure discharges and increases in cerebral γABA were not observed when solutions of sodium nitrite were injected into cats in amounts equivalent to NH_2OH. In 3 out of 4 samples there was no significant increase in cerebral γABA. Since sodium nitrite produced methemoglobinemia at least as extensive as that observed with NH_2OH, the results appear to rule out methemoglobinemia as a causative factor in electrographic changes. Actually, the methemoglobinemia appeared to be very mild and of short duration when doses of 10-20 mg/kg of NH_2OH were given. Use was not made of methylene blue to prevent methemoglobinemia because of the possibility of introducing complications in interpretation of EEG results. The preceding report(1) showed that methylene blue alone does not increase cerebral levels of γABA in rats.

From above data it is evident that NH_2OH can produce changes in neural excitability and content of γABA opposite to those induced by convulsant hydrazides(2,3). These findings are in agreement with a working hypothesis linking metabolism of γABA in a specific cerebral area to excitability of that area. Further work is in progress to extend the study of the anticonvulsant properties of NH_2OH, which may have important clinical value, and their mechanism of action.

Summary. Hydroxylamine when administered to immobilized cats in acute experiments in doses of 10-20 mg/kg markedly reduced duration and spread of electrically-induced afterdischarges while increasing cerebral levels of γ-aminobutyric acid. These findings increase the probability that a causal relationship may exist between metabolism of γ-aminobutyric acid in a particular cerebral area and its excitability.

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Further Studies on Flavonoids and Thymus Involution. (25107)

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Our recent report showed that feeding of quercetin, dihydroquercetin, and eriodictyol to rats induced thymus involution, mediated through the pituitary-adrenal axis(1). Results of similar studies with other flavonoids are here presented.

Materials and methods. Treatment of animals was similar to that of previous study(1), except that a different basal diet was used with the following percentage composition: degerminated corn meal 65, sucrose 10, casein 15, corn oil 4, U.S.P. No. XIV salt mixture 4, adequate vitamin mixture triturated in dextrose 2. This semi-synthetic diet was used in testing all flavonoids reported here, except in the case of morin where the non-synthetic diet used previously(1) was employed. Young female albino rats, 25-28 days old from our colony were fed the basal diet with or without addition of flavonoids for 12-14 days. The rats were then sacrificed, their organs examined carefully for gross changes and the fresh thymus weights recorded, except in the case of hesperidin and appropriate controls, where thymus weights were determined after fixation in formaldehyde. Naringenin, hesperetin, luteolin, morin, eriodictyol, and quercetin were fed at dietary level of 2%. Hesperidin and "Calcium Flavonate Glycoside, Lemon"†, which contains eriodictyol glycoside(2) as the main flavonoid component, as well as minor amounts of luteolin, quercetin and other glycosides (Horowitz, R. M., personal communication), were fed at level of 4%. Hesperidin and its aglycone hesperetin, naringin and morin were obtained from commercial sources. Naringenin was prepared from its glycoside naringin by acid hydrolysis. Since naringin is bitter, only the non-bitter aglycone naringenin(3) was tested. Luteolin was prepared from eriodictyol by the method of Lorette *et al.*(4). Quercetin and eriodictyol were prepared as previously reported(1).

Results based on fresh thymus weights expressed as % of body weights are summarized in Table I. Luteolin ($P<0.01$) and "Calcium Flavonate Glycoside, Lemon" ($P = 0.02$) caused significant thymus involution. The data on quercetin and eriodictyol confirm that reported previously(1) and provide a comparison with other data of the present report. Hesperetin, hesperidin, naringenin, and morin failed to elicit this response. No gross changes in rats receiving flavonoids could be observed at time of sacrifice. Growth of rats receiving flavonoids was comparable to that of those fed basal diets alone.

Discussion. Although final body weights in control and experimental groups were variable, especially in rats receiving eriodictyol, food intakes and rates of growth over the experimental period were comparable. Consequently thymus involution in certain experimental groups could not be due to the stress of anorexia.

A demonstrable thymolytic action by the flavonoids tested to date was observed only with those which possess a hydroxyl group on both the 3′ and 4′ positions of the flavonoid molecule (*cf.* quercetin, dihydroquercetin, eriodictyol and luteolin *vs.* hesperetin, naringenin and morin). On the other hand, a double bond between carbons 2 and 3 is not essential for the thymolytic action (*cf.* quercetin and luteolin *vs.* dihydroquercetin and eriodictyol); nor is the presence of a hydroxyl group on carbon 3 (*cf.* quercetin and dihydroquercetin *vs.* eriodictyol and luteolin). The structures of these flavonoids are shown in Fig. 1.

It is interesting that hesperetin, which is the 4′-methyl ether of eriodictyol, lacks the thymolytic action of the latter. It might be predicted that diosmetin, the 4′-methyl ether of luteolin, would also lack thymolytic action.

The relationship of the metabolic fate of

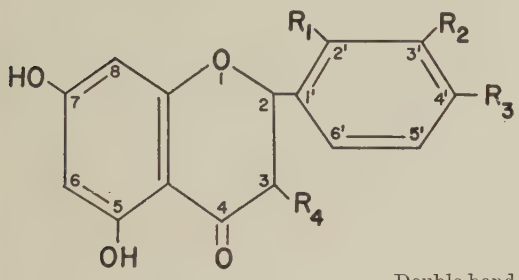
* Laboratory of Western Utilization Research and Development Division, Agric. Research Service, U. S. Dept. of Agric.

† A commercial preparation distributed by Sunkist Growers, Products Dept.

TABLE I. Thymus Gland Weights of Control and Flavonoid-Fed Rats.

Flavonoid fed	No. of rats	Range of final body wt, g	Mean body wt, g	Avg thymus wt, % of body wt		
				Mean ± S.E.	Diff. ± S.E.	P*
None (basal, semi-synthetic diet)	40	67–99	83.7	.350 ± .006		
Hesperetin	10	64–87	78.9	.340 ± .011	.010 ± .013	
Naringenin	10	77–90	84.7	.326 ± .017	.024 ± .018	
Luteolin	9	62–90	77.7	.313 ± .010	.037 ± .012	<.01
Ca-flavonate glycoside	10	74–101	85.2	.319 ± .014	.031 ± .012	.02
Eriodictyol	10	54–81	67.1	.295 ± .012	.055 ± .014	<.001
Quercetin	10	77–98	89.0	.303 ± .008	.047 ± .010	"
None (basal, non-synthetic diet)	5	65–90	79	.297 ± .07		
Morin (non-synthetic diet)	5	69–90	80	.306 ± .08	.009 ± .11	

* P = Probability that such a difference would occur by chance. Only significant values of P are indicated.



	R_1	R_2	R_3	R_4	Double bond 2:3
Hesperetin	H	OH	OCH_3	H_2	No
Naringenin	H	H	OH	H_2	"
Luteolin	H	OH	"	H	Yes
Eriodictyol	H	"	"	H_2	No
Quercetin	H	"	"	OH	Yes
Dihydro-quercetin	H	"	"	H, OH	No
Morin	OH	H	"	OH	Yes
Diosmetin	H	OH	OCH_3	H	"

FIG. 1. Generalized structure of flavonoids. Substituents for individual flavonoids are tabulated above.

various flavonoids to their thymolytic action will be discussed later.

Summary. (1) Hesperidin, hesperetin, naringenin, morin and luteolin were fed to young rats to determine their ability to induce thymus involution. Only luteolin was effective, and in this respect resembles quercetin, dihydroquercetin, and eriodictyol. (2) A commercially available preparation, "Calcium Flavonate Glycoside, Lemon," caused thymus involution. Some relationships between structure and thymolytic action of flavonoids are discussed.

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Demonstration of Glucose-6-Phosphatase in Mammalian Pancreas.* (25108)

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Previous workers demonstrated non-specific alkaline phosphatase in pancreatic ductules (1,2,3) and non-specific acid phosphatase in islets as well as in exocrine pancreas(4,5). Glucose-6-phosphatase has been demonstrated in liver, kidney and intestinal tract(6,7) but has been stated to be absent from pancreas (7). This enzyme is heat labile, inactivated at

* Supported by Grant from Nat. Science Fn., Washington, D.C.

pH 5 or below and under some conditions by formalin fixation(8,9). The enzyme is probably concerned with uptake and/or liberation of glucose by cells which contain it. Since the B cell responds to blood concentration of glucose(10,11) it seems reasonable to expect a mechanism controlling movement of glucose into and out of the cell. This viewpoint is, furthermore, consistent with the fact that glycogen deposits, found within pancreatic ductular epithelium and B cells under conditions of protracted hyperglycemia(12,13,14) recede quickly when blood sugar concentration reverts to normal(15). A study of pancreatic glucose-6-phosphatase was undertaken and its distribution compared with that of non-specific acid and alkaline phosphatase.

Material and methods. Pancreatic tissue was obtained from normal rabbits, guinea pigs and dogs and also portions of liver from rabbits. All animals were sacrificed by overdosage with nembutal. Tissues were removed immediately and frozen in petroleum ether, kept at –70° by bath of alcohol and dry ice (16). The frozen tissue was dried with filter paper and stored in stoppered tubes in deep freeze at –20°C. Cryostat sections at 5 or 10 μ were affixed to slides and stained immediately or were placed in neutral 6% calcium formol at –4°C for 15 minutes prior to staining. Additional blocks of pancreas were placed in Zenker-formol solution and processed for study of cell types(17). To demonstrate glucose-6-phosphatase activity, previously described technics(6,18) were modified. Slides were incubated for 10 to 30 minutes in a mixture consisting of 20 cc of 0.02 M solution of potassium glucose-6-phosphate, 20 cc of 0.2 M tris maleate buffer, pH 6.7, 3 cc of 2% lead nitrate and 7 cc of distilled water. Control sections were incubated without substrate as well as after iodine treatment and also at pH 5 and pH 9.2. For pH 5, acetate buffer was substituted for tris maleate buffer. Additional sections were incubated in the same substrate at pH 6.7 but with 20 cc of either 0.02 M B-glycero-phosphate, 0.02 M glucose-1-phosphate, 0.002 M adenosine phosphate or 0.002 M adenosine triphosphate substituted for glucose-6-phosphate. Non-specific acid and alkaline phosphatase were demonstrated at pH 5 and pH 9.2 respectively, utilizing B-glycero-phosphate as substrate as well as by azo dye method using alpha-napthyl-phosphate(19,20). For Zenker fixed tissue the periodic acid Schiff trichrome method(17) was used as well as modified aldehyde fuchsin trichrome method(21).

Results. In unfixed rabbit liver glucose-6-phosphatase activity was present only in cytoplasm of hepatic cells. No enzyme was present in blood vessels, canalicular system or Kupfer cells. After fixation in cold 6% calcium formol similar staining was obtained, with very slight, if any, diminution in enzyme activity (Fig. 1). This applied for pancreas as well, and since the tissue was better preserved after short formalin fixation this was used exclusively.

In the rabbit pancreas, with glucose-6-phosphate as substrate and 10 minute incubation, the precipitate was confined to B cell cytoplasm which frequently appeared to be more heavily stained in the perinuclear area (Fig. 2). Areas of islets presumably occupied by A and D cells did not stain (Compare Fig. 2 and Fig. 3). There was only very minimal diffuse staining of the exocrine parenchyma. Longer incubation times increased the precipitate in B cells but did not show staining at any other site. Control sections treated with Lugol's solution or incubated without substrate showed complete absence of staining. At pH 5 with 10 minute incubation there was markedly reduced B cell staining while at pH 9.2 almost no staining was observed.

Equivalent localization or intensity of staining as with glucose-6-phosphate was not obtained in the rabbit pancreas with any other substrate at pH 6.7 and 10 minutes incubation. Thus, with B-glycero-phosphate or alpha-napthyl-phosphate there was faint staining of the entire islet as well as the acinar tissue. Glucose-1-phosphate showed very faint islet staining and almost no staining of exocrine parenchyma. With adenosine phosphate there was very faint diffuse staining throughout. Adenosine triphosphate stained the cytoplasm and nuclei of islet and acinar cells. Cell borders and nuclear membranes stand out, as well as perivascular and periductular tissue. In addition, there was more intense

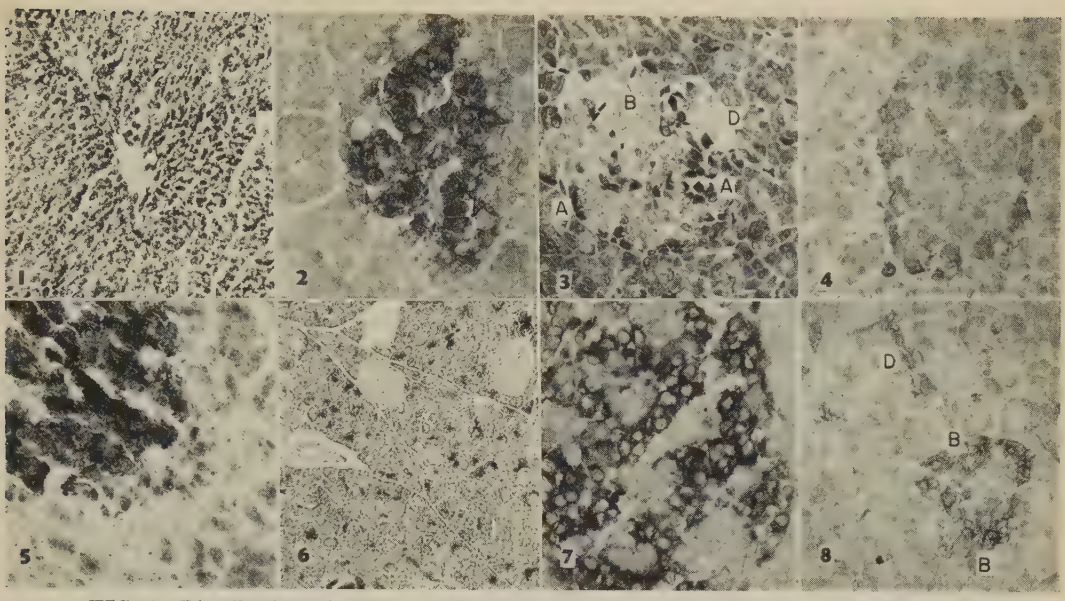

FIG. 1. Distribution of glucose-6-phosphatase in cryostat sections of rabbit liver fixed 10 min. in cold neutral 6% calcium formol, then incubated for 10 min. with glucose-6-phosphate at pH 6.7. Enzyme activity confined to cytoplasm of hepatic cells. × 32.

FIG. 2. Rabbit pancreas showing distribution of glucose-6-phosphatase. Staining almost entirely confined to B cells and more intense in perinuclear areas. × 183.

FIG. 3. Rabbit pancreas showing an islet with A, B and D cells. Periodic Acid Schiff Trichrome. × 183.

FIG. 4. Rabbit pancreas showing distribution of precipitate when adenosine triphosphate is substituted for glucose-6-phosphate in incubation mixture. Intense staining of peripheral islet cells, which are either A or D cells. × 125.

FIG. 5. Rabbit pancreas showing distribution of non-specific acid (B-glycero-) phosphatase at pH 5 after 45 min. incubation. Activity is present throughout pancreas. Staining greatest at vascular pole of islet cells and at secretory pole of acinar cells. × 183.

FIG. 6. Rabbit pancreas showing distribution of non-specific alkaline (B-glycero-) phosphatase at pH 9.2 after 45 min. incubation. Activity present mostly in ductular system. × 48.

FIG. 7. Guinea pig pancreas showing distribution of glucose-6-phosphatase. Staining confined almost entirely to B cells. × 183.

FIG. 8. Dog pancreas showing distribution of glucose-6-phosphatase. Marked staining present in cytoplasm of ductular epithelium (D) and in B cells (B). × 125.

staining of groups of islet cells which, from their localization, are thought to be A or D cells (Fig. 4). Non-specific acid phosphatase was found throughout rabbit pancreas when either B-glycero-phosphate or alpha-napthyl-phosphate was used at pH 5. Its activity was greatest, however, at the secretory poles of acinar cells and at vascular poles of islet cells (Fig. 5). Non-specific alkaline phosphatase as demonstrated by either method was mostly present in the ductular system (Fig. 6).

In guinea pig pancreas distribution of glucose-6-phosphatase was the same as in rabbit (Fig. 7). In the dog, however, staining was also present in cytoplasm of ductular epithelial cells (Fig. 8).

Discussion. Glucose-6-phosphatase activity has been demonstrated within the cytoplasm of pancreatic B cells in rabbit, guinea pig and dog. In the dog, in addition, some activity also was observed in ductular epithelium. Specificity of histochemical localization of this enzyme is demonstrated by the differences in distribution or the limited activity when rabbit pancreas was incubated at pH 6.7 for equally short times with other substrates and by the fact that maximum staining was obtained at optimum pH for this enzyme with loss of activity at pH 5 and pH 9.2(22,23). Inability of a previous worker (7) to demonstrate glucose-6-phosphatase activity in pancreas may have been due to differences in incubation mixture or to the fact

that unfixed frozen pancreatic tissue may autolyze rapidly when incubated.

Contrary to reports that formalin inactivates glucose-6-phosphatase(6,7,9) we found that short fixation in cold neutral 6% calcium formol does not appreciably alter its activity or distribution and gives better tissue preservation.

The known relationship of insulinogenesis to blood sugar concentration(10,11) suggests that B cell glucose-6-phosphatase might function in relation to the mechanism of insulin release. Perhaps the net amount of glucose-6-phosphate available for B cell metabolism is the controlling factor in limiting rate of insulin output. The net rate of formation of glucose-6-phosphate would depend on relative velocities of hexokinase and glucose-6-phosphatase reactions and velocity of glucose phosphorylation by hexokinase would depend upon blood sugar concentration. Thus an increase in blood sugar concentration or decrease in glucose-6-phosphatase activity would increase available glucose-6-phosphate and increase insulin output. Conversely, lowering blood sugar or increasing glucose-6-phosphatase would diminish available glucose-6-phosphate and also diminish insulin output.

Summary. Glucose-6-phosphatase has been demonstrated in pancreatic B cells of rabbit, guinea pig and dog, and in canine ductular epithelium. Short fixation of fresh frozen cryostat sections in cold neutral 6% calcium formol does not inhibit its activity or alter its distribution in liver or pancreas. Furthermore, specificity of histochemical reaction for glucose-6-phosphatase was demonstrated by differences in staining when other substrates were incubated at same pH for equal time. In view of known relationship of blood sugar concentration to rate of insulin secretion, a hypothesis is advanced relating presence of this enzyme in B cells to control of insulin secretion. It is suggested that net rate of glucose-6-phosphate formation is the controlling factor and that this would be influenced by any increase or decrease of glucose-6-phosphatase activity.

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Effect of Corticosteroid Excess and of Bilateral Adrenalectomy on Metabolism of Mg^{28}.* (25109)

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There is little evidence indicating a clear and direct relationship between adrenal steroids and metabolism of divalent ions. For magnesium ion, the main route of excretion from the body is *via* the kidneys, once this ion has entered the circulation. In adrenal insufficiency it has been reported(1) that magnesium blood levels are elevated. It would appear that this hypermagnesemia is the result of deficient renal excretion. There is only fragmentary evidence which indicates that as a result of adrenal steroid insufficiency certain magnesium dependent tissue enzymes may become altered and result in specific metabolic derangement. This latter evidence would imply either an altered tissue concentration or an altered tissue state for the magnesium ion (2). There is no work reported which indicates any conclusive relationship (or lack of relationship) between magnesium ion and elevated levels of adrenal cortical steroids in animals. With availability of an isotope of magnesium of suitable half-life, we investigated in rats, whether, when compared to the normal, either hyper or hypo adrenal cortical state altered tissue distribution and plasma disappearance of this isotope following its intravenous administration as a single dose.

Materials and methods. A group of 200 female rats of approximately 200 g were used. Of these animals, 100 were used as normal controls; 65 were given 2 mg hydrocortisone daily for one week and 40 rats were bilaterally adrenalectomized 3 weeks before study and maintained on their usual diet plus glucose-salt-bicarbonate mixture added to their drinking water. Under light ether anesthesia, the normal and steroid treated animals received between 0.2 to 0.3 μc Mg^{28} intravenously *via* a tail vein and were then sacrificed serially over the following 2 or more hours. Blood was immediately drawn from the inferior vena cava, and the total heart, total liver, and sample of skeletal muscle taken for assay. The adrenal insufficient animals were handled in the same way, except that they received no anesthesia.‡ Two ml samples of plasma, the total heart, total skeletal muscle sample, and a 2 ml aliquot of an acid digest of the liver was counted. All data are expressed in terms of concentration of Mg^{28}/ml of plasma or gram of tissue as percent of administered dose of isotope. Total heart and liver weight were determined on the basis of percent of body weight(3). Total muscle samples were weighed and counted in tared stoppered counting tubes. The method of assay, calibration and counting isotope in plasma and tissue were the same as previously described except that in these studies tissue was not dried to constant weight(4).

Results. Fig. 1 A, B, and C show plasma disappearance and uptake of isotopic magnesium by liver, heart, and skeletal muscle in normal rats following a single intravenous injection. The plasma disappearance curve is similar to that noted in dogs(4). At dose levels used in these rats, uptake of isotopic magnesium by liver, heart and skeletal muscle is rapid and is sustained over 2 hours of observation. During period of observation the turnover of isotope is apparently quite slow. Again the striking difference in avidity of heart muscle as compared to skeletal muscle is noted. Uptake of Mg^{28} by livers of these rats/g of tissue) is slightly higher than heart muscle.

In the steroid treated animals no deviation

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‡ Ether anesthesia had no influence on Mg^{28} distribution or dynamics, since a number of normal and steroid treated rats were studied without prior anesthesia and were identical to those which had received anesthesia.

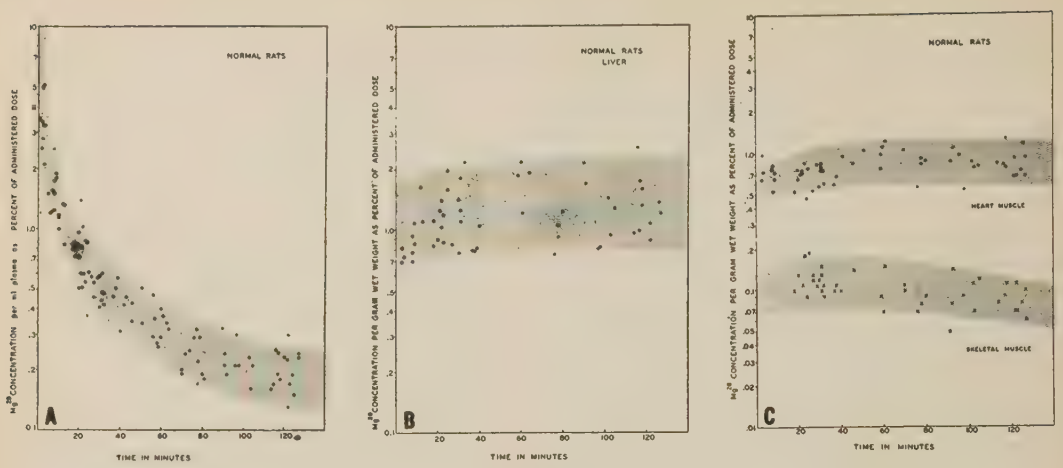

FIG. 1. Normal rats. A. Plasma disappearance of Mg^{28} following a single I.V. inj. B. Uptake of Mg^{28} by liver. C. Uptake of Mg^{28} by heart and skeletal muscle. Shaded areas in A, B and C represent 95% confidence limits for normal data.

from the normal is detectable, as shown in Fig. 2 A, B, C.

No significant deviation from the normal for plasma disappearance of Mg^{28} or its uptake by heart and skeletal muscle is noted in bilaterally adrenalectomized rats (Fig. 3, A, B, C). Uptake of isotope by livers of bilaterally adrenalectomized rats is slow in early periods but reaches normal levels after one hour.

Discussion. For the parameters measured, the data in these normal rats are similar to those obtained in dogs receiving a single intravenous injection of isotopic magnesium. In this respect, the above rat data are merely confirmatory.

With regard to the finding that adrenal steroid excess did not alter plasma disappearance and tissue uptake of Mg^{28} during 2 hours observation, there are 2 possible explanations: (1) at normal steroid levels an optimum effect is already present which additional steroid will not alter and (2) there is no direct relationship between adrenal glucocorticoids and the body's handling of magnesium. In this study we are unable to differentiate definitely between these 2 explanations, both of which appear equally plausible. It would be of interest to study the effects of excess aldosterone on Mg^{28} dynamics since renal loss of magnesium has been reported in cases of primary aldosteronism(5). Apparently from these studies glucocorticoids do not affect the dynamics of the magnesium ion.

With regard to bilaterally adrenalectomized rats maintained without steroid supplementation, no deviation from normal plasma disappearance curve could be detected. In dogs receiving a large single dose of isotopic magnesium intravenously, as much as 30% of administered dose is excreted *via* the kidneys within the first hour(4). Any significant alteration in renal function resulting in inability to excrete magnesium normally as a result of adrenal insufficiency should result in some deviation in magnesium dynamics. In our rats, adrenal insufficiency did not alter the plasma disappearance curve from the normal. This would suggest either that renal excretion was normal or that there must be a higher tissue concentration of the ion to account for the normal plasma curve. Except for a somewhat delayed uptake of Mg^{28} by the liver, tissue concentrations of various soft tissues studied were normal. It may therefore be concluded that in this short term study in rats, adrenal insufficiency in no way altered the dynamics of magnesium disappearance or distribution. Further, if renal function was actually depressed (it was not measured) as a result of adrenal insufficiency, apparently compensation was made here to maintain all measured parameters within normal limits.

It is realized that there is hazard in extra-

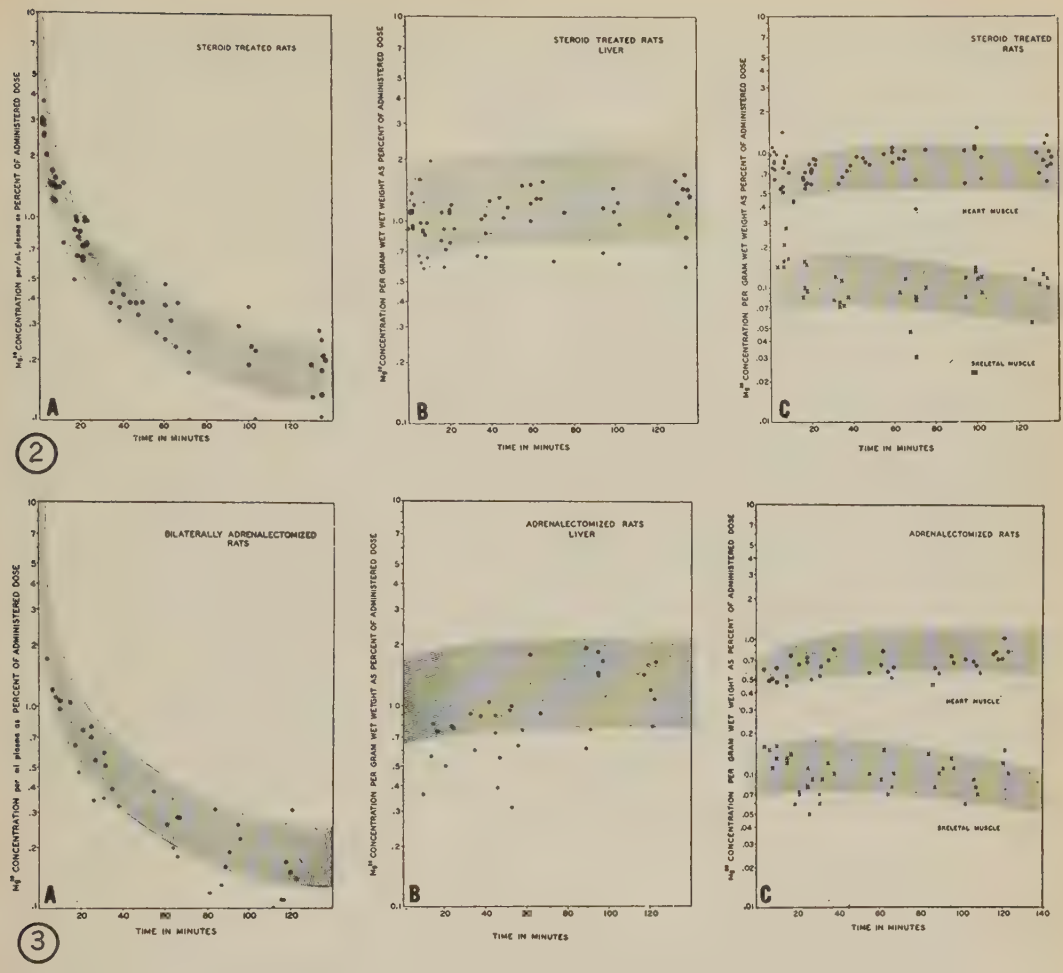

FIG. 2 and 3. Plot of these data is similar to Fig. 1. Shaded area is 95% confidence limit for normal data of Fig. 1.

polating from acute short term studies to long term chronic studies. No statements are warranted from this report relative to chronic experiments which attempt to evaluate effects of long term hyper or hypo-adrenal cortical states to magnesium metabolism. It appears that acutely neither hyper nor hypo-adrenal function alter magnesium metabolism as defined in these studies.

Summary. (1) The plasma disappearance and soft tissue uptake of a single i.v. dose of Mg^{28} was studied in normal rats over a 2 hour period. These results were compared to identical studies in rats given large doses of cortisol and rats made adrenal insufficient. (2) No deviation from normal could be detected in cortisol treated or adrenal insufficient rats.

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Effect of Cortisone on Ethionine-Induced Pancreatitis in the Rat. (25110)

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Beneficial results observed following cortisone in treatment of pancreatitis in man have led to its recommendation as a therapeutic adjunct in this disease(1,2). On the other hand, pancreatic lesions have been produced in rabbits by cortisone(3,4) and similar changes have been noted at post-mortem in humans receiving ACTH or cortisone(5). Also a decrease in pancreatic exocrine secretion following ACTH and adrenocortical steroids in man has been described(6). A study of cortisone in experimentally produced pancreatitis, therefore, seemed warranted. Furthermore, pancreatic pathology induced by ethionine is thought to result from alterations in methionine metabolism(7,11) and the effects of cortisone in protein metabolism gave particular interest to its use in ethionine pancreatitis.

Methods. Female rats of Wistar strain were utilized. All were allowed a Purina Chow diet consisting of 5% fat, 23% protein, 44% carbohydrate, 6% fiber and 9% ash, as desired, throughout the experiment. Cortisone and ethionine were injected into the peritoneal cavity. All animals were weighed at beginning and at termination of experiment. When rats were sacrificed and the viscera inspected, microscopic sections were prepared of pancreas, liver and stomach. Pathologic changes were arbitrarily graded from 1 to 3, according to severity. Initially, the effect of cortisone alone was studied. Five rats, averaging 200 g in weight, received 2 mg of cortisone daily for 7 days and were sacrificed on the eighth. Two similar groups of 5 rats each received 7 and 15 mg of cortisone and sacrificed on eighth day. In evaluation of ethionine and cortisone, 10 rats averaging 168 g received 0.6 mg/g of body weight of ethionine daily for 7 days and sacrificed on eighth. A second group of rats of same weight were given the same amount of ethionine, and in addition each rat received 7 mg of cortisone daily and sacrificed on eighth day. A third experiment was carried out similar in all ways to the 7 day evaluation of ethionine and ethionine plus cortisone, except that rats were sacrificed on third day. In the 2 day evaluation, the group given ethionine plus cortisone consisted of 9 rats.

Results. Rats receiving cortisone only continued in good health during the injections. Gain in weight during the 7 day period averaged 9 g/rat. There was no correlation of weight gain with dosage of cortisone used. There was considerable variation in amount of weight gained and some rats at each cortisone dosage level either failed to gain or lost weight during the experiment. Except for more darkly staining basophilic cytoplasm in the acinar cells, no alterations in pancreatic structure could be noted on microscopic section. Gross or microscopic abnormalities were also

TABLE I. Pathologic Changes in Rats Receiving Ethionine and Ethionine-Cortisone for 7 Days.

Animal No.	Drug	Microscopic pathology graded 1-3: Pancreas	Liver	Stomach	Avg wt loss, g
	Ethionine,				
1	.6 mg/g	2	3	0	71
2	"	3	1	0	67
3	"	3	1	Ulcer	66
4*					
5*					
6*					
7	"	3	3	0	39
8†		3	2	Ulcer	51
9	"	3	1	0	65
10	"	3	2	Gastritis	49
	Idem + cortisone, 7 mg				
11	daily	3	3	Ulcer	43
12†		3	2	Gastritis	56
13	"	3	3	Ulcer	58
14†		2	1	"	57
15	"	3	1	"	47
16	"	1	1	"	61
17†		3	1	Gastritis	59
18†		3	2	Ulcer	53
19	"	3	2	0	36
20	"	3	1	Ulcer	53

* Died on 3rd day. † Died on 8th day.

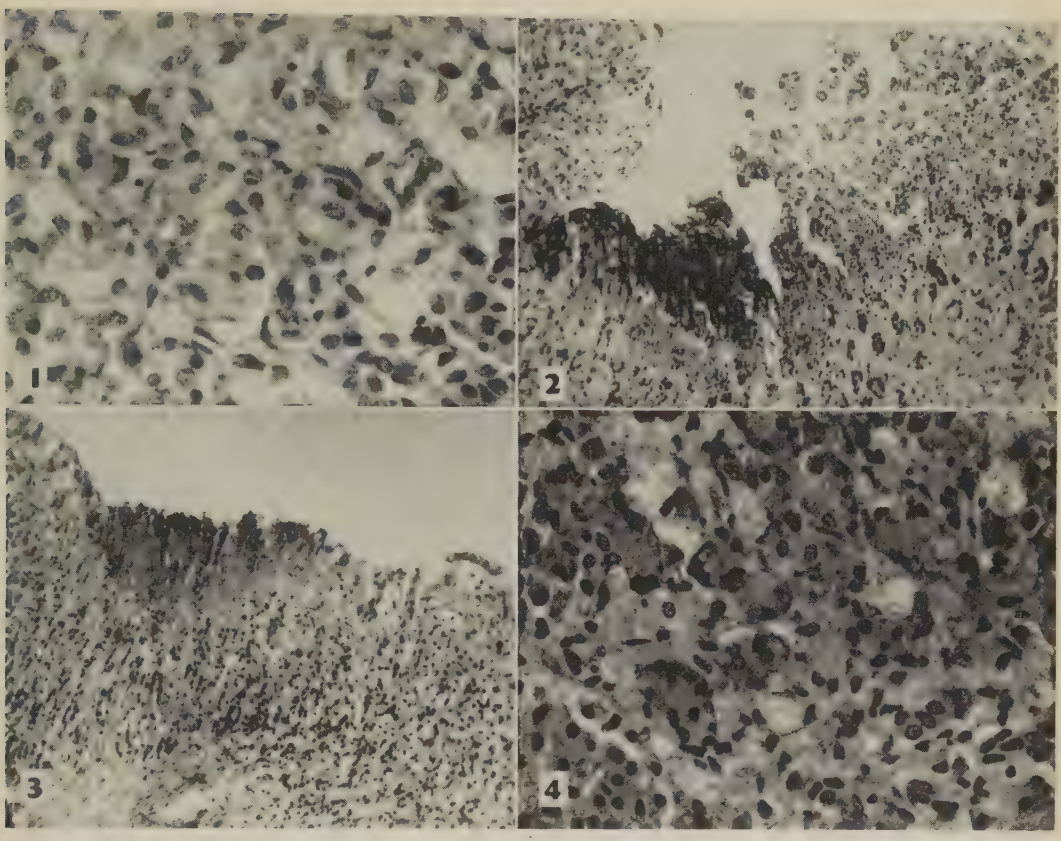

FIG. 1. Grade III pancreatic lesion.
FIG. 2. Gastric ulcer in rat receiving ethionine and cortisone for 7 days.
FIG. 3. Gastritis in rat receiving ethionine and cortisone.
FIG. 4. Grade I pancreatic lesion in rat receiving ethionine and cortisone for 2 days.

absent in livers and stomachs of these animals.

Results in rats receiving ethionine only and ethionine plus cortisone for 7 day period appear in Table I. Animals 4, 5 and 6 died on third day and autolysis of tissue made evaluation of histologic changes impossible. Rats 8, 12, 14, 17 and 18 died on eighth day; *i.e.*, the day they would have been sacrificed. Gastric ulceration with hemorrhage was found in each. An average weight loss of 58.5 g/rat over the 7 day period was noted in those receiving ethionine only. Those receiving ethionine and cortisone lost 52.3 g/rat. All rats appeared ill by third day; their fur was ruffled and stained, their eyes were red, their food intake was markedly reduced, and they showed little spontaneous activity. The hepatic and pancreatic lesions seemed identical both in character and severity in rats receiving ethionine and those receiving ethionine and cortisone. Pathologic changes which occurred in the pancreas are demonstrated in Fig. 1, which shows a lesion considered Grade III in severity. The pancreas showed marked disturbance of architectural pattern. Normal acini were rare and the acinar cells showed loss of basophilic cytoplasm. Proliferation of young fibroblastic tissue and infiltration with mononuclear cells were prominent in each section. Pancreatic blood vessels appeared dilated and congested but intra-pancreatic hemorrhage was minimal. Vacuolization of varying degree was present. Liver pathology consisted of congestion and periportal infiltration with mononuclear cells. Fibroblastic proliferation of mild degree was found in the periportal area in some sections. Hepatic cell necrosis or fatty infiltration was conspicuously absent.

The gastric mucosa was somewhat less than normal in thickness in all rats. A hemor-

TABLE II. Pathologic Changes in Rats Receiving Ethionine and Ethionine-Cortisone for 2 Days.

Animal No.	Drug	Microscopic pathology graded 1-3: Pancreas	Liver	Stomach	Avg wt loss, g
	Ethionine,				
1	.6 mg/g	1	0	0	2
2	"	1	0	0	6
3	"	1	0	0	0
4	"	1	0	0	12
5	"	1	0	0	0
6	"	1	0	0	4
7	"	1	0	0	1
8	"	1	0	0	5
9	"	1	0	0	7
10	"	1	0	0	7
				Avg wt loss	4.4
	Idem + cortisone, 7 mg				
11	daily	1	0	0	3
12	"	1	0	0	6
13	"	1	0	0	11
14	"	1	0	0	1
15	"	1	0	0	8
16	"	1	0	0	5
17	"	1	0	0	5
18	"	1	0	0	1
19	"	1			1
				Avg wt loss	4.1

rhagic gastritis or shallow gastric ulceration was found in 3 of 7 rats given ethionine only, and in 9 of the 10 rats receiving cortisone in addition. Figs. 3 and 4 are illustrations of the gastritis and gastric ulcers found. The ulcers were small, discrete, often multiple and occurred in both squamous and glandular portions of the stomach. All animals with such gastric lesions had black guaiac-positive material in the stomach and small bowel. The 5 animals dying of effects of drugs had gastric lesions. Four of these had been given cortisone and ethionine, and one only ethionine.

There was no correlation between weight loss and presence or severity of lesions. There appeared to be no correlation between severity of pancreatic lesions and presence of gastric lesions.

Results in animals receiving ethionine and ethionine plus cortisone for a 2 day period appear in Table II. All of these lived to termination of experiment. They appeared healthy and their appetite remained good throughout. An average weight loss of 4.3 g/rat during the 2 day period occurred. There was no significant difference in weight loss of those receiving ethionine from those receiving both drugs. Livers and stomachs appeared normal grossly and on microscopic examination. Pancreatic changes are shown in Fig. 4. These were considered Grade I, consisting of loss of basophilic cytoplasm and some disturbance in acinar arrangement. The pancreatic lesions were of equal severity in those receiving ethionine only and in those receiving ethionine and cortisone.

Discussion. The nature of metabolic abnormality produced by ethionine is not entirely clear. An inhibition by ethionine of the hepatic uptake of radioactive sulfur from labeled methionine has been demonstrated(10). In a similar study of pancreatic metabolism, an accelerated rate of methionine incorporation was found(11). In each case, the metabolic abnormality and the associated hepatic or pancreatic structural change were prevented by additional methionine. The overall catabolic effect of cortisone on nitrogen metabolism is accompanied by actual increase in liver protein and in serum protein(12). This result indicates that the alteration in protein metabolism produced by cortisone does not provide utilizable methionine in sufficient amounts to prevent development of ethionine lesions.

Death of the 3 ethionine-treated rats on third day of the planned 7 day experiment, is unexplained. If cortisone had a protective effect during the first days of ethionine administration which was dependent upon modification of the pancreatitis, some amelioration of the pancreatic lesion would have been expected in rats sacrificed on third day. No significant difference in severity could be found at this time. The anti-inflammatory effect of cortisone would not seem to be of great importance inasmuch as neutrophilic infiltration and edema were not prominent in ethionine lesions.

The high incidence of gastric lesions with hemorrhage and the probable part they played in the demise of 5 rats is of great interest. Alterations in gastric mucosa have been reported(13,14) after ethionine but ulcerations with hemorrhage have been infrequent in reports of ethionine-induced pancreatitis. Although the numbers are small, the 90% inci-

dence of gastric ulceration in those receiving cortisone as well as ethionine as compared to the less than 50% incidence in those given ethionine only, suggests that cortisone accelerates development of these lesions. Neither the mode of production of gastric lesions nor the mechanism by which cortisone influences their development, is apparent. The high rate of metabolic activity in the gastric mucosa as in the pancreas is presumably an important factor in explaining the vulnerability of these organs to injury by ethionine. Prolonged administration of adrenal corticoids(15) and hypothalamic stimulation(16) increased gastric acid and pepsin secretion, and cortisone may aggravate ulcer formation in this manner.

Summary. Typical pancreatic changes were induced by ethionine, which were not altered by addition of cortisone. The high incidence of gastric ulceration and hemorrhage found with ethionine alone was doubled in rats receiving cortisone also.

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Relationship Between Inactivation of Poliovirus by Phenol and Appearance of Ribonuclease-Labile Infectivity.* (25111)

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Gierer and Schramm(1) found that infectivity remaining after treatment of tobacco mosaic virus with phenol could be destroyed with ribonuclease in contrast to the stability to ribonuclease of original untreated virus. Similar results were subsequently reported for Mengo encephalitis virus(2), poliovirus(3,4, 5), West Nile encephalitis virus(3), Eastern equine encephalomyelitis virus(6), and Semliki Forest virus(7). In the above studies aqueous virus preparations were serially extracted with phenol. Our results of an investigation of some factors involved in use of phenol for obtaining ribonuclease-labile infectivity from poliovirus are given below.

* Aided by grant from National Fn.

Materials and methods. Polioviruses. Wild type virus of the Brunhilde strain (antigenic type 1) and of the Akron strain (type 1) and the cr^i mutant(8) of Akron were used. *Reagents.* Solutions of phenol saturated with water at 0° were prepared; these were 8.03 M phenol (a) by titration with standard NaOH and (b) by quantitative organic hydroxyl determination(9). Solutions of phenol were also prepared by dissolving a weighed amount of phenol, dried over P_2O_5, to volume in high-cystine (0.20 mM L-cystine) altered Eagle's

maintenance medium ($hcAE_m$)(8). The pH was adjusted with HCl and/or NaOH while bringing to volume. Phenol concentration in such a solution was calculated from phenol determinations performed on similarly dried and weighed samples of phenol by above methods (a) and (b). Solutions of crystalline ribonuclease (Nutritional Biochemicals Corp., Cleveland, O.) at 0.10 mg/ml in water were prepared. *Treatment of virus with phenol.* Water-saturated phenol solutions were used for tests in which virus was mixed with a relatively small volume of phenol solution whereas solutions of phenol in $hcAE_m$ were used for tests in which the relative volume of phenol solution was large. In the former case phenol concentration in the reaction mixture is expressed as $\frac{100v_p}{v_p + v_v}$ per cent water-saturated phenol where v_p is volume of water-saturated phenol mixed with v_v volume of virus stock, while in the latter case the molar concentration is given; molar concentration = (0.0803) (per cent water-saturated phenol). Temperature was 0°; where reported, pH was determined immediately after starting and just before stopping the reaction. Timing was as follows: exposure was started by rapidly and thoroughly mixing virus and phenol; exposure was stopped by adding approximately equal volume of ethyl ether at 0° and mixing vigorously. The ethereal phase was discarded and the aqueous phase was extracted twice more with cold ether. Residual ether was removed with N_2. To the virus was added one-ninth volume of ribonuclease solution (see above). This was then incubated 15-30 minutes at *ca* 26°. Ribonuclease was replaced by water in the control. Kidney cells from cynomolgus monkeys (*Macaca irus*) or rhesus monkeys (*M. mulatta*) were dispersed with trypsin using essentially the technic of Youngner(10) and were grown in 60 mm diameter petri plates on lactalbumin growth medium of Melnick(11). Kidney cell cultures were maintained during viral action on altered Eagle's maintenance (AE_m) medium(8) containing L-cystine at 0.050-0.20 mM; this medium was used without agar for formation of stocks to be treated with phenol and with agar for plaque formation.

Results. Phenol concentration. In early experiments with 4% water-saturated phenol in reaction mixture great variation in the extent of drop in plaque titer from mixture to mixture was encountered. Testing a wide range of phenol concentrations indicated that, with this method of mixing the virus with small volume of concentrated phenol, 4% is a critical concentration (Fig. 1). With this method, however, some inactivation may have been due to presence in parts of mixture, during mixing, of phenol concentrations higher than the final. This was tested using the method of mixing the virus with a relatively large volume of dilute phenol in $hcAE_m$ (Table I); these data indicate that inactivation due to momentarily high phenol concentrations during mixing virus with concentrated phenol is of appreciable magnitude. (Compare Table I with Fig. 1, especially 4.97% with 5%, respectively; the somewhat greater variation from mixture to mixture encountered in Fig. 1 was probably due to (a) variation in magnitude of this special inactivation at mixing time, (b) low accuracy in measuring volume of concentrated phenol mixed with virus, and (c) variation in pH, which may have varied as much as from 5 to 7½.) The data in Table I show accurately and quantitatively the dependency of inactivation on concentration.

Reaction mixtures were sampled after exposure times of 0.5 to 240 minutes. Over this range the surviving virus fraction in a mixture was constant (Fig. 2). At pH's 4 ± 0.2, 6.2 ± 0.2, and 7.4 ± 0.2 the minimal phenol concentration required to drop the titer to 10^{-3} of the original was 0.36, 0.40, and 0.44 M, respectively (estimated from data in Table I).

Ribonuclease-labile infectivity. The low (ca $<10^{-5.5}$ of original) phenol-surviving virus fractions, but neither the original virus nor the high surviving fractions, could be inactivated by ribonuclease. This ribonuclease-labile infectivity was present 0.5 minute after start of reaction and its titer was essentially constant to at least 240 minutes after the start.

Discussion. The population of infectious Brunhilde poliovirus particles used for experi-

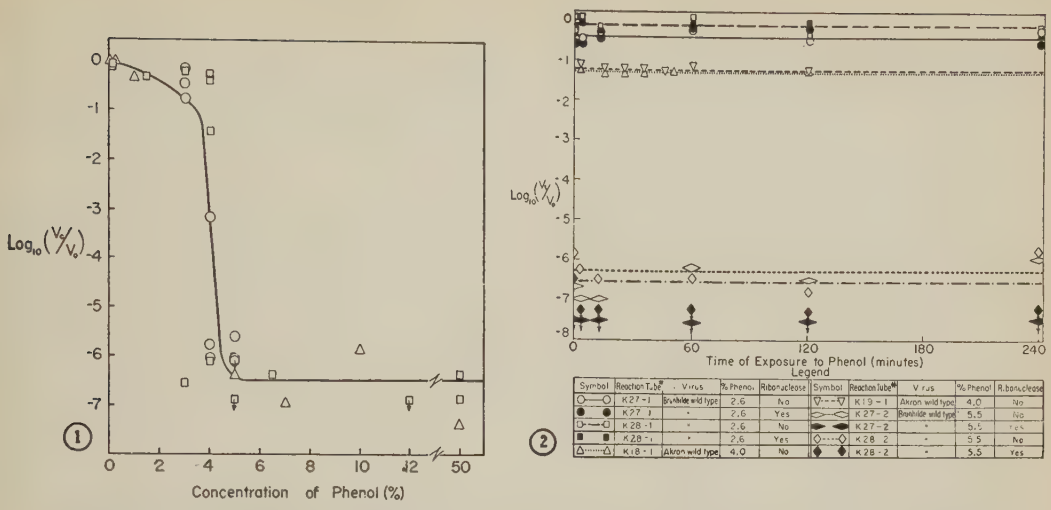

FIG. 1. Inactivation of poliovirus at 0° by phenol as a function of concentration of phenol, using the method of mixing the virus with relatively small volume of water-saturated phenol. V_c is titer (plaque-producing particles/ml) after exposure to concentration c of water-saturated phenol for 20 min.; V_o is titer without exposure to phenol. Each point represents a different reaction tube and data are from 3 experiments, △ for experiment K10 using the Akron *cr*$^+$ mutant, ○ for K22 using Akron wild type virus, and □ for K26 using Brunhilde wild type. An arrow (↓) indicates that no virus was found, a point so indicated thus representing maximal titer. No ribonuclease was used. In reaction tubes with water-saturated phenol at concentrations *ca* >7% the aqueous phase was saturated with phenol (diphasic reaction mixture).

FIG. 2. Lack of dependence of inactivation of poliovirus by phenol and of titer of ribonuclease-labile infectivity present after treatment with 5.5% water-saturated phenol on time of exposure at 0° to phenol in range of about 0.5 to 240 min. The method of mixing the virus with relatively small volume of water-saturated phenol was used. V_t is titer (plaque-producing particles/ml) after time t of exposure to phenol; V_o is titer without exposure to phenol. An arrow (↓) indicates that no virus was found, a point so indicated thus representing a maximal titer.

ments summarized in Table I was heterogeneous in phenol-stability. Though no particle in this population was completely phenol-stable, some were stable at higher concentrations than were others; for example, at pH 7.4 ± 0.1 with 0.40 M phenol, about 1% of

TABLE I. The Inactivation of Poliovirus at 0° by Phenol as a Function of Concentration of Phenol and of pH of Reaction Mixture.

Phenol concentration, M: Added to virus	Phenol concentration, M: Final in reaction mixture	% water-saturated phenol* in reaction mixture	pH during reaction	$\text{Log}_{10}(V_c/V_o)$: Exp. 1	Exp. 2	Exp. 3, Tube 1	Exp. 3, Tube 2	Arithmetic mean
.298	.295	3.67	4.0 ± .2	−1.84	−1.52	−1.34	nd	−1.57
			6.2 ± .2	− .52	− .36	− .03	”	− .30
			7.4 ± .1	− .46	−1.72	−2.14	”	−1.44
.404	.399	4.97	4.0 ± .2	−4.87	−4.58	−(>5.29)	”	−(>4.91)
			6.2 ± .2	−3.72	−3.10	−2.95	”	−3.26
			7.4 ± .1	−2.07	−2.30	−1.83	”	−2.07
.500	.494	6.15	4.0 ± .2	−2.20†	−(>5.18)	−(>5.71)	−(>5.71)	−(>5.53)
			6.2 ± .2	−5.17	−(>4.97)	−(>5.71)	−(>5.70)	−(>5.39)
			7.4 ± .1	−5.35	−4.94	−(>5.71)	−5.40	−(>5.35)

* Included to facilitate direct comparison with Fig. 1 and 2.

† Probably technical error: not included for calculating mean.

nd = not done. V_c and V_o as in Fig. 1. Method of mixing the virus with relatively large vol of solution of phenol in hcAE$_m$ was used. No ribonuclease was added. Wild type Brunhilde virus was used throughout. Controls without phenol were kept under same conditions at 3 pH's; they suffered no loss of infectivity.

the infectious Brunhilde particles were stable. If at least the major part of the phenol-stable ribonuclease-labile poliovirus infectivity is *produced* by phenol treatment, as it apparently is in the case of Mengo encephalitis virus(3), then the finding that this infectivity is present in its maximal titer (maximal for assay system employed) within not more than 0.5 minute, while the original ribonuclease-stable virus population disappears within not more than this same period of time, suggests that both this disappearance and the appearance of the phenol-stable ribonuclease-labile infectivity may be the result of a single fast reaction.

Summary. The influence of phenol concentration, time, and pH on phenolic inactivation of poliovirus at 0° was studied. A minimal phenol concentration of about 0.5 M was required for removal of all the original ribonuclease-stable poliovirus infectivity. This minimal requirement was influenced slightly by pH; it was lower at pH 4.0 than at pH 6.2 and lower at pH 6.2 than pH 7.4. Amount of inactivation was independent of time of exposure to phenol over range of 0.5 to 240 minutes. Poliovirus populations studied were heterogeneous with respect to phenol-stability, some particles requiring higher phenol concentrations than others for inactivation. Under conditions giving phenolic inactivation of all ribonuclease-stable infectivity, the ribonuclease-labile infectivity was present 0.5 minute after start of reaction and its titer remained essentially constant for at least 4 hours.

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Pharmacological Studies with Nialamide, A New Antidepressant Agent. (25112)

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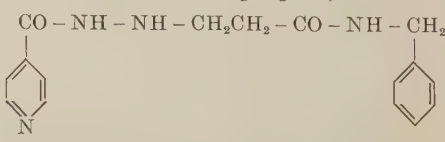
The introduction of monoamine oxidase (MAO) inhibitors as antidepressants has opened up a new concept in chemotherapy of mental disorder. As a result of favorable modification of psychic state of patients during its clinical trial as a tuberculostat, iproniazid was investigated intensively in depressed patients(1) and had marked antidepressant properties. Since side effects such as postural hypotension(2) and hepatotoxicity(3) were encountered, it was necessary to search for agents which lacked these attributes but retain the therapeutic value. From a large series of carboxamido alkyl hydrazines(4) investigated for this purpose, nialamide,*



was chosen for further studies because its out-

* Generic name of Niamid, Chas. Pfizer & Co.

standing *in vivo* and *in vitro* MAO inhibitor potency and lack of side effects(5). We present some important pharmacological studies with nialamide, with particular emphasis on its antidepressant activity.

Materials and methods. 2,900 male Swiss Webster mice, 40 rabbits, 50 cats and 40 dogs were used. Although a causal relationship between MAO inhibition and antidepressant action has not been established, there is close correlation of these in some of the agents currently used to treat depressed psychotic patients. We therefore chose the following measures for assessing activity of nialamide as an antidepressant: antagonism of reserpine depression, potentiation of 5-hydroxytryptophan (5-HTP) and MAO inhibition *in vivo*. *Reserpine antagonism.* Male albino mice weighing 17 to 25 g were pre-treated with MAO inhibitor either orally or intraperitoneally followed 18 hours later by intraperitoneal injection of reserpine, 5 mg/kg. Three and 4 hours after reserpine injection, the amount of ptosis and decrease in spontaneous motor activity of animals was scored. Degree of reserpine antagonism was calculated from the ratio of difference between score for reserpine control and that for the pre-treated mice, compared to the former, expressed as percent. A similar procedure was followed with cats and dogs. In these animals, the MAO inhibitor was given orally, and dose of reserpine, 1 mg/kg, given intravenously. *Pyretogenic response to 5-HTP.* An alternative means of measuring MAO inhibitory effect was suggested by Horita and Gogerty(6) using potentiation by MAO inhibition of the pyretogenic response of rabbits to 5-HTP. Rabbits were pre-treated by subcutaneous injection of MAO inhibitor 18 hours before intravenous injection of 5-HTP, 25 mg/kg. Rectal temperature was taken at 30 minute intervals both before and on day of test. Nialamide had a negligible effect on body temperature. Only the highest dose, 32 mg/kg, caused a significant increase (0.41°C, $P = 0.04$) over the previous day. Following injection of 5-HTP, peak temperature occurred in about 3 hours. *In vitro* analysis of dog brain stem for MAO activity and for serotonin and norepinephrine content was done by modification of the Bogdanski method(7). Monoamine oxidase activity in this tissue was assayed using serotonin as substrate. Tissues were taken from animals which received daily doses 5 days or 6 months. The time course of action of nialamide was followed quantitatively by determining degree of protection by nialamide against reserpine facilitation of Metrazol for 11 days. Albino mice were infused intravenously with 0.5% solution of Metrazol at 0.05 ml/10 sec until onset of tonic convulsions(8). Reserpine, 5 mg/kg injected intraperitoneally 3 hours prior to Metrazol infusion, lowers the threshold to Metrazol-induced convulsions to approximately 37% of control value. The ability of MAO inhibitors to potentiate hexobarbital sleeping time was determined in albino mice. Animals were injected with the inhibitors 18 hours before, 1 hour before, or simultaneously with intraperitoneal injection of hexobarbital, 100 mg/kg, and duration of sleeping time determined. Loss and recovery of righting reflex was taken as onset and end point of sleep.

Results. Symptomatology. Nialamide alone caused few overt symptoms in mice. Normal animals treated orally or parenterally in dose range to 100 mg/kg showed remarkably little effect attributable to the drug. A dose of 100 mg/kg caused slight decrease in spontaneous motor activity for about 60 minutes followed by mild increase lasting about 24 hours. At 200 mg/kg the effect was somewhat more noticeable but not marked. Intraperitoneal and oral LD_{50} with 95% confidence limits in mice were 742 (584-942) and 1,000 (741-1358) mg/kg, respectively(9,10). At toxic doses irritability, tremors, and salivation were observed, all of which are manifestations of sympathetic stimulation.

Reserpine antagonism. To compare the antidepressive potency of nialamide with that of iproniazid and other agents, reserpine antagonism was used. Previous administration of monoamine oxidase inhibitor postponed the onset and in some cases altered the character of depression resulting from reserpine. The intensity of this action was proportional to dose. Nialamide had about 2 to 3 times the

FIG. 1. Reserpine effect in cats following MAO inhibition. N, 18 hr after nialamide, 5 mg/kg, P.O.; I, 18 hr after iproniazid, 40 mg/kg, P.O.; C, control, no treatment; N1, I1, C1: 1.5 hr after reserpine, 1 mg/kg, I.V. N5, I5, C5: 5 hr after reserpine, 1 mg/kg, I.V.

potency of iproniazid in dogs and about 10 times in mice and cats (Fig. 1).

Potentiation of pyretogenic action of 5-HTP. Horita(11) used potentiation of the pyretic response to 5-HTP to compare potencies of MAO inhibitors. This procedure was used to compare potencies of iproniazid and nialamide (Fig. 2). By this test nialamide was about 2.5 times more potent than iproniazid.

In vitro analysis was done for serotonin and norepinephrine content as well as monoamine oxidase activity of brain stem tissue from dogs given nialamide, iproniazid, or β-phenylisopropylhydrazine (PIH). Differences in potency observed in intact animals correlated with degrees of inhibition of the enzyme and elevation of serotonin content (Fig. 3, Table I). From these results a difference in enzymatic action between nialamide and the others became evident. Whereas they all caused elevation of serotonin, nialamide also increased norepinephrine content. Brain stem tissue of dogs examined after 6 months continuous dosage *i.e.*, 1 to 30 mg/kg/day showed the same changes in amine content and enzyme activity indicating very little cumulative effect with prolonged treatment (Table I).

TABLE I. Serotonin and Norepinephrine Content of Dog Brain Stem following Nialamide.

Inhibitor	Length of treatment	Daily dose, mg/kg P.O.	Brain stem amine content 5 HT, μ/g	NE, μ/g
Controls	5 days		.52	.32
Nialamide	"	50	1.0	.61
PIH	"	10	2.8	.26
Iproniazid	"	50	1.0	.35
Controls	6 mo		.55	.21
Nialamide	"	1	.57	.28
"	"	3	.57	.25
"	"	10	1.25	.50
"	"	30	1.54	.52

Duration of action. The degree of reserpine antagonism due to a 10 mg/kg dose of nialamide reached a maximum in about 1-2 hours, then fell slowly over a period of days, a significant portion of initial activity still present

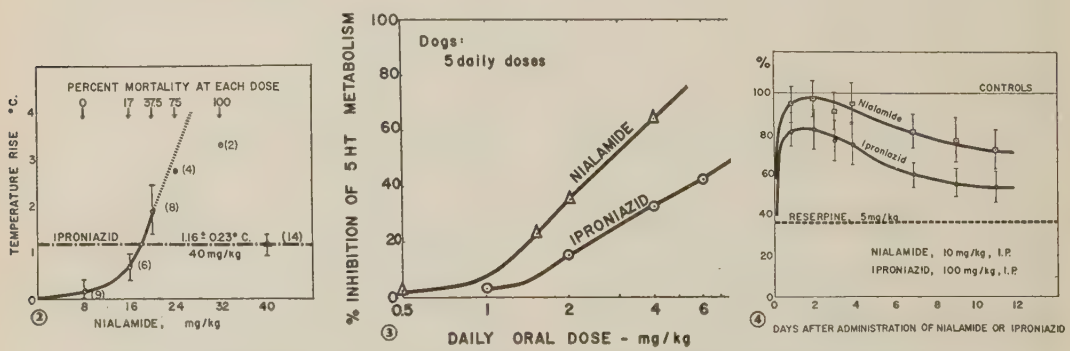


FIG. 2. Potentiation of 5-HTP pyretogenesis; peak temp. rise ± S.E., about 3 hr after inj. of 5-HTP, 25 mg/kg, I.V.; nialamide, S.C., 18 hr before 5-HTP; No. of rabbits/dose in parentheses.

FIG. 3. Brain MAO inhibition.

FIG. 4. Duration of action, protection against reserpine facilitation of Metrazol-induced convulsions, vertical axis: % of tonic convulsant volume of Metrazol for controls; Metrazol, I.V., 0.5% solution, 0.05 ml/10 sec.; reserpine, I.P., 5 mg/kg, 3 hr before Metrazol infusion; 12 mice/treatment; vertical bars indicate stand. error.

TABLE II. Effect of Nialamide on Hexobarbital Anesthesia.

Treatment*	Inj. time†	Duration‡ LRR, min.	Ratio T/C§	△ means, min.‖	$^{s}\triangle$, min.	t	d.f.	P
Nialamide + hexo.	Simult.	70.7	2.86	46.0	3.6	12.7	22	$<<0.001$
Hexobarb. control		24.7						
Nialamide + hexo.	1 hr	44.6	3.0	29.9	4.85	6.2	18	"
Hexobarb. control		14.7						
Nialamide + hexo.	16 "	31.4	.8	−10.1	4.86	2.1	18	0.06
Hexobarb. control		41.5						
Iproniazid + hexo.	Simult.	132.6	6.9	113.5	7.3	15.5	22	$<<0.001$
Hexobarb. control		19.1						
Iproniazid + hexo.	1 hr	95.4	7.6	82.8	5.1	16.3	18	"
Hexobarb. control		12.6						
Iproniazid + hexo.	18 "	15.6	.7	−6.1	1	6.1	18	"
Hexobarb. control		21.7						
Nialamide¶ + hexo.	Simult.	121.7	4.8	96.6	10.2	9.5	22	"
Hexobarb. control		25.1						

* Nialamide, 10 mg/kg, IP; iproniazid, 100 mg/kg, IP; hexobarbital, 100 mg/kg, IP. † Time between inj. of inhibitor and hexobarbital. ‡ Mean duration of loss of righting reflex. § Ratio of mean duration of treated to that of control. ‖ Difference between mean duration of treated and control. ¶ Nialamide, 100 mg/kg, IP.

after 12 days (Fig. 4).

Hexobarbital potentiation. Potentiation of hexobarbital sleeping time by iproniazid has been reported by Fouts and Brodie(12). Inhibition of oxidation of the side chain is the mechanism proposed. Plaa, Evans, and Hines(13) utilized potentiation of pentobarbital by a series of halogenated hydrocarbons as indication of degree of interference with liver function and found good correlation with histologic signs of toxicity. With this in mind, nialamide and iproniazid were compared for hexobarbital potentiation at doses which produced equivalent degrees of reserpine antagonism, 10 and 100 mg/kg respectively. Whereas iproniazid prolonged sleeping time 6.9 times, nialamide potentiation was only 2.9 times that of controls (Table II). That this effect was unrelated to monoamine oxidase inhibition was suggested by difference in time of reserpine antagonism and hexobarbital potentiation, the latter reaching a peak about 1 hour after administration and essentially absent 18 hours later (in preparation, R. P. Rowe, *et al.*)

Discussion. The antidepressant potency of nialamide has been demonstrated to be greater than that of iproniazid as judged by reserpine antagonism, 5-HTP potentiation, and *in vitro* monoamine oxidase inhibition. Although difference in activity between nialamide and iproniazid varied from species to species, the former was always more active. Similar potency of parenteral and oral dosage indicates good absorption of nialamide.

The most important aspect of this study concerns the correlation of animal tests with clinical results. No proof is available as yet implicating a causal connection between monoamine oxidase inhibition and antidepressant activity in the human. However, it has been observed that in humans monoamine oxidase inhibition may be demonstrated with nialamide(14) at approximately the same dose that gives a therapeutic effect in depressed patients (15).

Iproniazid and other monoamine oxidase inhibitors raised the serotonin content of dog brain, while norepinephrine levels were not significantly altered in this species. However, nialamide also caused an elevation of norepinephrine. Whether this factor is in any way related to lack of hypotensive activity of nialamide reported in humans(14,15) has not been answered.

Other differences between nialamide and iproniazid are demonstrable. Thus, the prolonged hexobarbital sleeping time after iproniazid contrasts with mild potentiation of this phenomenon with nialamide. Prolongation of hexobarbital sleeping time has been utilized as an indication of interference with liver func-

tion and consequent toxicity. This is in accord with chronic toxicity experiments in dogs (10) where nialamide had no functional, gross anatomical, or histological effects on the liver.

Summary. Nialamide was a potent monoamine oxidase inhibitor by both *in vitro* and *in vivo* test. Its potency relative to that of iproniazid varies from 3:1 to 12:1 depending on test and species used. Nialamide was also shown to be qualitatively different from other MAO inhibitors in some of its pharmacological actions, factors which may explain absence of both liver toxicity in dog and postural hypotension in man.

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Experimental Detection of Left-to-Right Circulatory Shunts with Injections of Krypton^{85}. (25113)

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Improved methods for detection of circulatory shunts have evolved in recent years. Dye-dilution curves and inhaled foreign gas technics(1,2,3) have both proved superior to oximetry in detection and localization of left-to-right shunts. Our experience has demonstrated the usefulness of solutions of radioactive krypton gas (Kr^{85}) in detection and localization of right-to-left shunts. By continuously monitoring the expired air with a Geiger-Müller tube, appearance time and concentrations of krypton^{85} in expired gases can be determined. When Kr^{85} is injected into the right side of heart or pulmonary artery, it appears immediately and in high concentrations but when injected into the left side of heart the appearance time is long and initial concentration is low. This observation suggested that the early appearance of Kr^{85} in expired air after its injection into the left side of heart would be indicative of a left-to-right shunt.

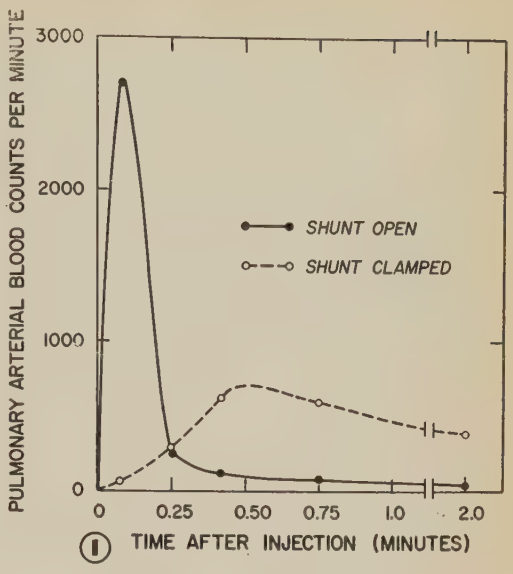
Materials and methods. Left-to-right shunts were constructed in 8 mongrel dogs by anastomosis of the left subclavian artery to the left pulmonary artery. Anesthesia was induced with sodium pentobarbital, a cuffed-endotracheal tube was inserted and positive pressure respiration was maintained at a rate of 20/min by a demand respirator. Seven dogs were studied in acute experiments and one was catheterized 2 weeks after operation. A #6 Cournand catheter was inserted *via* femoral artery to the root of the aorta. Injections of krypton^{85} in saline solution were made at the aortic valve with the shunt open and after it was occluded. Patency of anastomosis was

proved in every instance by presence of a continuous thrill in the pulmonary artery and by postmortem examination. Aortic root injections were also performed in 2 control animals without shunts. Radioactivity of expired air was recorded from a Geiger-Müller tube inserted into the airway between the endotracheal tube and respirator. Integrated counts of expired air were made at 10 second intervals for the first 60 seconds after injection and at one minute intervals for the next 3 minutes. The background count over a 10 second interval was determined immediately before each injection. In 2 animals integrated samples of pulmonary arterial blood were also drawn at 10 second intervals for the first 2 minutes after injection. These samples were counted as whole blood in a continuous gas-flow G-M tube. In another animal, radioactivity of expired air was recorded continuously with a count-rate-meter and direct-writing oscillograph. The krypton^{85} solution was prepared by injecting 3.1 mc of Kr^{85} into a 30 ml vial containing equal amounts of normal saline and air. Three to 5 ml of resulting solution, containing approximately 30 μc of Kr^{85} were used for each injection and the volume of saline replaced.

Results. Forty-four Kr^{85} injections were made in 10 dogs without functioning shunts. The integrated count of radioactivity in expired air for the first 10 seconds after injection never exceeded 75 counts above background. Fifty-four injections were made in 8 dogs with patent subclavian-pulmonary anastomoses. The count in this group for the same interval ranged from 136 to 624 counts above background. In subsequent time intervals no clear separation of the 2 groups was present.

When the shunt was open, Kr^{85} appeared immediately in pulmonary arterial blood and its concentration was maximal in the first 10 seconds (Fig. 1). When the shunt was occluded, the gas appeared in low concentration during the first 10 seconds, and reached its peak concentration at 40-50 second interval. The relationship between time-concentration curves of Kr^{85} in pulmonary arterial blood and expired air is shown in Fig. 2.

Continuous recordings of radioactivity in expired air are shown in Fig. 3. When the shunt was occluded appearance time after aortic root injection was 12 seconds and build-up time was 20 seconds. When the shunt was patent, however, appearance time was 4 seconds and build-up time only 11 seconds.



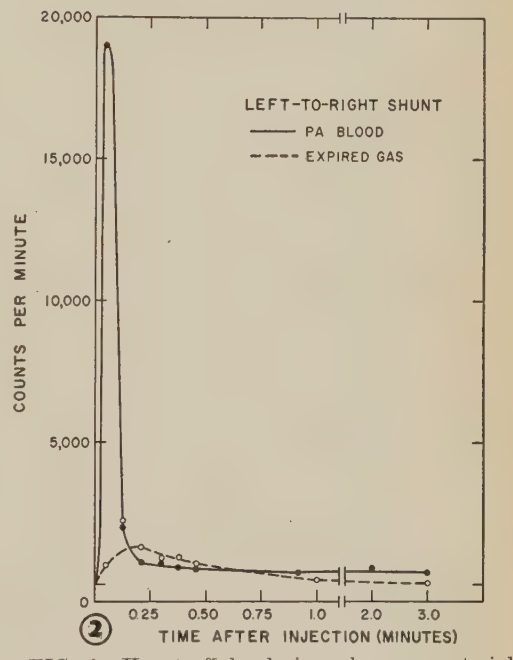


FIG. 1. Krypton^{85} levels in pulmonary arterial blood after aortic root inj.

FIG. 2. Comparison of expired air and pulmonary arterial levels of Kr^{85} after aortic inj. proximal to a patent shunt.

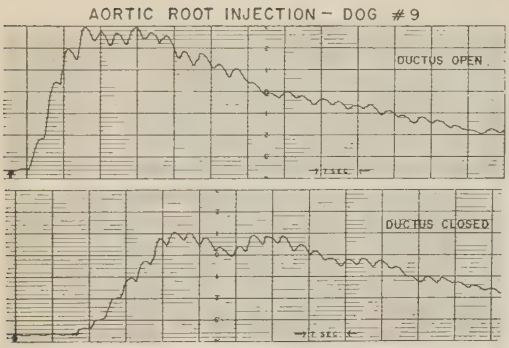


FIG. 3. Direct recordings of radioactivity of expired gas after inj. of Kr^{85} at aortic root.

Discussion. When Kr^{85} in solution is introduced directly into the pulmonary artery, it appears in expired air within 2 seconds. Thus, expired air closely reflects presence of the isotope in pulmonary arterial blood and obviates the need for sampling blood from the pulmonary artery. Detection of a left-to-right shunt by this method depends upon appearance time in expired air. An accurate estimate of this time is best obtained by continuous recording of radioactivity in expired air (Fig. 3).

When Kr^{85} is injected into the left heart or aorta of a normal animal, appearance time is 10 seconds or more; when a left-to-right shunt is present the gas reaches the pulmonary artery almost immediately and appearance time is 5 seconds or less. A basis is thus established for detecting the presence of a shunt. By selective injection at various locations in the left heart and aorta, the site of the shunt may be determined.

Preliminary clinical experience with this method in patients with congenital heart disease indicates that it may offer distinct advantages over present methods of detecting left-to-right shunts.

Summary. Left-to-right shunts were constructed in 8 mongrel dogs by anastomosis of left subclavian artery to left pulmonary artery. When solutions of krypton^{85} were injected into the root of the aorta proximal to a functioning shunt, the krypton gas could be detected in the airway by a Geiger-Müller tube in less than 5 seconds. When similar injections were made in the absence of a shunt, the appearance time was usually 10 seconds or more. Thus, by monitoring expired gas it is possible to detect the presence of a shunt resulting in early appearance of Kr^{85} in pulmonary arterial blood following injection into the left side of heart or the aorta.

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Localization of Myosin in the Conduction Bundle of Beef Heart. (25114)

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Although the characteristic striated fibrillar structure of cells of the conduction bundle of mammalian heart suggests that these fibrils contain protein similar to those of other muscle tissue, the identity of the contractile substance in this area has not been investigated. By means of Coons fluorescent antibody technic Finck, Holtzer and Marshall(1) and Klatzo, Horvath and Emmart(2) have shown that myosin is localized in the A band of striated muscle fibers of both skeletal and heart muscle. Using this immunological technic rabbit antimyosin globulin conjugated to fluorescein has been applied to sections of the conduction bundle of beef heart to determine presence of this contractile protein in the fibrils. The chemical isolation and identity of muscle proteins of the conduction bundle will be reported elsewhere.

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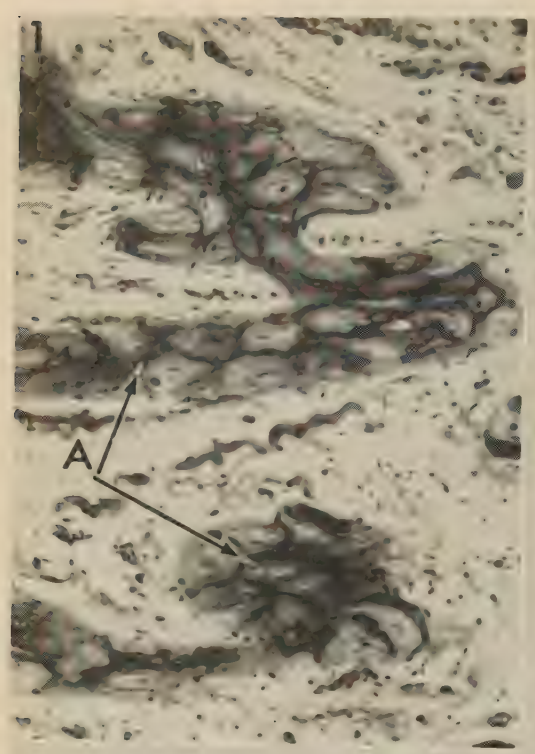
Materials and methods. The antigen. Myosin from calf heart was prepared from ventricles of heart after fat, connective tissue and vessels were removed. In addition, the conduction system was dissected away as far as this could be followed macroscopically. The hearts were obtained while still warm and chilled at once to 0°C and kept cold under dissection. The tissue was ground twice, washed once with water and twice with 0.05 M KCl. It was then extracted twice while stirring with 2 volumes 1.1 M Kl plus 0.1 M phosphate buffer, pH 7.4(3). The supernatant fluids were recovered by centrifugation after each extraction and combined. These latter extracts were diluted with water to concentration of 0.025 M to precipitate the myosin. The precipitate was dissolved by adding M NaCl to final concentration of 0.75 M. This solution was then centrifuged to remove impurities, and further purified by 3 precipitations. The final myosin precipitate was dissolved in 0.67 M NaCl and stored, refrigerated, at +2°C. In this solution nitrogen content was determined by either biuret or Kjeldahl methods and protein concentration held at about 3 mg/ml. Myosin prepared from skeletal muscle was identical with that previously used(2). *Production of antisera.* Myosin prepared from heart muscle was administered intravenously to rabbits 3 times weekly in doses of 8 mg in 2.6 ml of 0.67 M NaCl. After 8 weeks of administration antimyosin sera was obtained. The potency of the antiserum was determined by precipitin reaction *in vitro,* and the globulin fraction reassayed after conjugation to fluorescein isocyanate. *Procedure of staining.* When serum of sufficiently high antimyosin titer was obtained, the γ globulin fractions were conjugated to either fluorescein isocyanate or isothiocyanate and purified as previously described(2,4). The fluorescent antibody solution was applied for 20 minutes directly to the frozen, freshly cut tissue sections without previous fixation. The excess antiglobulin solution was quickly washed away with 4 or more changes of cold .15 M saline adjusted with phosphate buffer to pH 7.4 and the tissue mounted in neutral glycerin diluted 50% in .15 M saline and examined under ultra violet light. Fluorescent normal rabbit globulin was prepared and used for control "staining." In addition, freshly cut sections of all tissues were mounted directly in glycerin without staining and examined for native fluorescence and these fluorescent areas identified by comparison with formalin fixed tissue sections stained by various methods. *Tissue of conduction bundle.* The so-called "bundle of His" or conduction bundle in these studies, was dissected from beneath the endocardium of right ventricle of beef heart within 15-20 minutes after death and frozen immediately in isopentane chilled to −140°C with liquid nitrogen. Frozen tissue was stored in vials packed in dry ice until sectioned. Sections were cut at 7 μ in a cryostat at −18°C.

Results. The histology of the conduction bundle of the mammalian heart has been described in detail by Todd(5), Schiebler(6,7) and others(8,9). Area of the bundle of beef heart in this study includes only the region close to the atrioventricular node and part of bundle of right ventricle. Preparations of this part of the bundle stained with phosphotungstic acid haematoxylin reveal part of the remaining endocardium with dense strands of collagen, reticulin and elastic fibers of the subatrial tissue. Below this compact area these fibers form a dispersed mesh imbedded in ground substance within which blood vessels, nerves and root-like fibers of the Purkinje system are immeshed. Near the node, where branching is extensive, the fibers may be 8 or more cells thick while smaller fibers are only 2 or 3 cells wide (Fig. 1). Sections of this area "stained" with fluorescent antimyosin globulin solution show that only the fibrils of the bundle absorb and retain the fluorescent antibody (Fig. 3). This indicates the specificity of the antibody for the protein of myofibrils of the bundle.

Morphology of the Purkinje cell differs markedly in various areas of the conduction bundle but the basic integrity of the cellular pattern, the characteristic cytoplasmic structures and the structural pattern of the cross striated myofibrils have been clearly defined by means of electron microscopy(10,11,12). These studies suggest that myofibrils composed of fine fibrils are dispersed mostly in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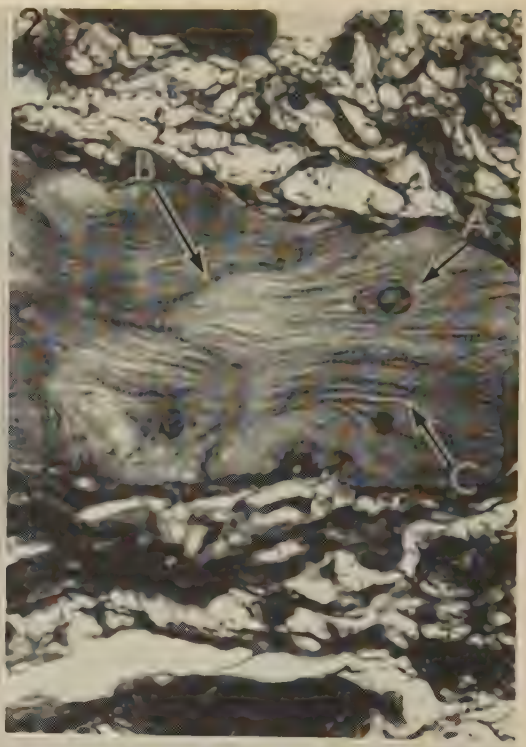
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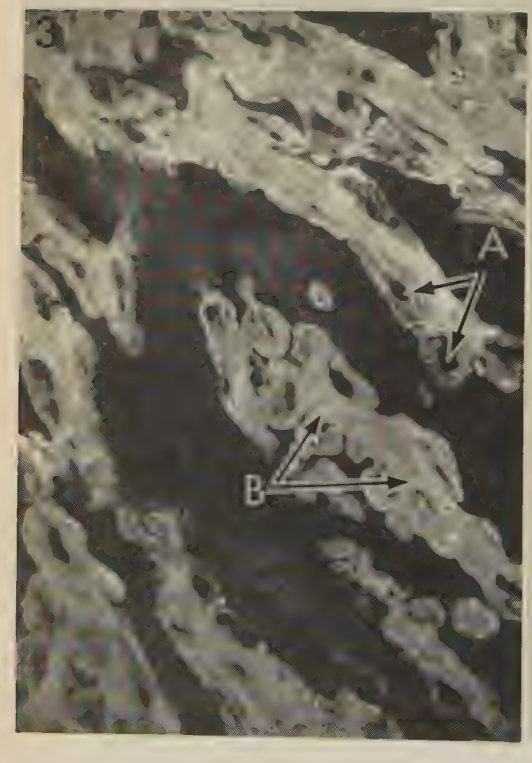
3
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the peripheral area of the cytoplasm. When sections of the bundle were stained with fluorescent antimyosin solution the center of cell surrounding the nucleus remains essentially unstained (Figs. 3 and 4) with an occasional myofibril penetrating between or near the nuclei in multinucleated cells (Fig. 4-B).

In sections of the conduction bundle of beef heart fixed in 10% formalin and stained with peracetic aldehyde fuchsin, following staining procedure of Fullmer and Lillie(13), the cross striations of myofibrils are clearly discernible (Fig. 2-C). "Staining" of similar sections with fluorescent antibody to myosin demonstrated cross striations of myofibrils (Fig. 4-E), and show clearly the similarity of myofibrils of the conduction bundle and those of the cardiac muscle(2). When fibrils are in a contracted state the bands of myofibrils which extend under the sarcolemma give the cells a scalloped border (Fig. 4). Purkinje cells of the fibrils are intercepted at intervals by intercalated discs with foliated borders which are composed, in part, by transverse myofibrils, and which stain positively with antimyosin solution (Fig. 4-D).

In freshly prepared tissue, strands of collagen, outside the fiber have a native blue fluorescence which, like all fluorescence, appears white in the print (Fig. 4-A). This fluorescence, native to the tissue, fades rapidly and is not seen in frozen tissue kept for several days. In all preparations other fibrillar elements of connective tissue show no evidence of antibody tagging, indicating that such impurities as may be present either in the antigen or fluorescent antibody solution do not interfere with immunological binding or specificity of antibody for myosin.

Discussion. Identification of the contractile protein, in myofibrils of the Purkinje cell as myosin, is dependent upon the antigenicity of myosin and the immunological reaction of the antimyosin globulin solution for specific proteins composing the myosin molecule. Kamikozawa (1923) demonstrated that positive seriological reactions could be obtained with crude extracts prepared from the His bundle of beef heart and rabbit sera immunized against this extract(14). However, His preparations were too crude to justify any conclusions as to identity of proteins present. It was not until 10 years later that the contractile protein of muscle tissue became known as myosin(15,16,17). Within the last 10 years it has been demonstrated that myosin isolated from either skeletal or cardiac muscle is antigenic in animals of different species(18, 19). Since it has been shown that myosin is composed of actin and tropomyosin(20,21, 22) it seems probable that antisera from rabbits injected with myosin possess multiple antibodies to the contractile protein and that in our study these antibodies probably function as a unit in binding to the antigen. Holtzer and Abbott(23) using fluorescent antimyosin globulins on embryonic muscle cells *in vitro* have shown that appearance of cross striations of myofibrils is concurrent with contractility. Changes in contractile proteins which may occur in cell division and maturation need to be followed in greater detail. Variation in histology of different areas of the conduction bundle together with its large cellular unit and dispersed myofibrils affords a unique opportunity for the study of localization of contractile proteins. The results of such experiments will be reported elsewhere.

Summary. 1) Positive binding of fluorescent antimyosin solution to myofibrils of the

FIG. 1. Fibers of conduction bundle (beef heart) stained with phosphotungstic acid haematoxylin (A), × 150.

FIG. 2. Several Purkinje cells of fiber stained with peracetic acid aldehyde fuchsin, Halmi stain (Fullmer and Lillie): (A) nuclei, (B) cell wall, (C) myofibrils showing cross striation, × 300.

FIG. 3. Fluorescence photograph of fibers of conduction bundle—"stained" with fluorescent antimyosin globulin. (A) area surrounding nucleus, negative; (B) positive staining (fluorescence) of myofibrils in Purkinje cells, × 150.

FIG. 4. Fluorescence photograph of several Purkinje cells "stained" with fluorescent antimyosin globulin. (A) collagen fibers in surrounding connective tissue possess a blue fluorescence; (B) area of nucleus; (C) myofibrils in outer cytoplasm; (D) intercalated disc; (E) striated myofibrils; (F) contracted myofibrils along outer sarcoplasm give scalloped appearance, × 500.

Purkinje cell demonstrates presence of myosin in myofibrils of the conduction bundle of beef heart. 2) Antibody to myosin whether prepared from skeletal muscle or heart tissue is capable of binding heart muscle antigen.

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The Fetal Lung, A Source of Amniotic Fluid. (25115)

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The physico-chemical features of amniotic fluid in last third of pregnancy are very similar to those found in interstitial fluid(1), particularly in animals in which the allantoic sac is still present during this period, *e.g.*, goats and sheep(2). Amniotic fluid could therefore be produced by an ultrafiltration process. This production requires an organ with large surface supplied by many capillaries, in which hydrostatic pressure is higher than colloid-osmotic pressure of plasma proteins (14-20 mm Hg in goat fetus(3)). Two organs present such properties: kidneys and lungs. Hydrostatic pressure in pulmonary capillaries should be higher than colloidosmotic pressure, as pressure in pulmonary artery is relatively high, due to the nature of fetal circulation. Pressure in the pulmonary artery is higher than that found in the aorta and peripheral arteries, where pressure reaches values of about 70-80 mm Hg(4) at end of pregnancy. The kidney cannot be the only source of amniotic fluid inasmuch as amniotic fluid is present also when the fetal urethra is not patent and the urine must pass through the urachus into the allantoic sac, as in goats and sheep, to 100-120 days pregnancy, *i.e.*, to its last third. This research investigated the possible production of fluid by the lungs of fetus and to measure its rate of flow.

Methods. Experiments were performed on goats and guinea pigs during last third of pregnancy. The goats lightly anesthetized with chloralose (70 mg/g intravenously) were

TABLE I. Fluid Production from Lung of Goat Fetuses and Physico-Chemical Features of Bronchial and Amniotic Fluid.

Fetal age, days	Fetal wt, g	Period of fluid production, hr	Vol of fluid production, ml	Flow, ml/kg/hr	Osmotic pressure, mOsmol		Protein, % content	
					Bronchial fluid	Amniotic fluid	Bronchial fluid	Amniotic fluid
158	755	2	.11	.073	302	304	.1	.2
117	1070	2	.59	.280	304	289	.2	.2
118	1460	*			310	293	.2	.2
105	900	*			295	300	.2	

* Flow of liquid from the trachea was large in first 10 min. (to 20 ml), and then ceased.

placed, in supine position, in a bath of Ringer solution at 38°C. After opening the abdomen the position of the fetus was found, and the head delivered. Keeping the head under Ringer solution, the trachea was cannulated. These operations were performed under water to prevent air from entering into airways of fetus. The tracheal cannula was connected to glass tubing by a polyethylene catheter filled with saline. The glass tubing was positioned at the same level of liquid in which the fetus was placed, to counterbalance the hydrostatic pressure of the saline solution above the fetus. After replacing the head of the fetus, the uterus was sutured. Loss of amniotic fluid during the operation was reduced to a small amount. At end of experiment the osmotic pressure of amniotic fluid and of the liquid contained in the bronchi, was measured by freezing point technic, and protein content was determined by a refractometric method. Guinea pigs were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). A small incision was made in wall of uterus above the fetal trachea. The trachea was cannulated and connected to a glass tube without delivering the head of the fetus. Uterus and abdomen were then sutured. All these operations were performed under saline solution. Displacement of liquid in the respiratory apparatus could be measured by movements of the meniscus in the glass tubing.

Results. Four experiments were performed on goats.* In 2 experiments fluid came out of the trachea continuously for 2 hours. In the other 2, the flow stopped after about 10 minutes. During this time a large amount of fluid was produced (Table I). Liquid coming out of trachea was clear, colorless and the osmotic pressure and protein content was similar to those found in amniotic fluid.

Ten experiments were performed on the guinea pig, and there was no evidence of fluid entering the trachea. The flow of liquid out of the trachea during first 5 minutes was disregarded as probably due to a change in lung volume.

In 4 experiments the flow of liquid from the trachea was negligible (0 to 0.05 ml) and hence has not been reported. In the remaining 6 experiments the liquid came out for 30 to 240 minutes with a flow of 1.3 to 6.7 ml/kg/h.

Discussion. In some experiments on the guinea pig the amount of liquid that flowed slowly and continuously from the trachea was larger than total amount of fluid that could be contained in lungs at beginning of experiment. This finding demonstrates that the fetal lung produces fluid which is similar to amniotic fluid.

These experimental results support the hypothesis of Jost and Policard(5). These authors observed an increase of lung volume of rabbit fetus after tying the trachea, and they thought that amniotic fluid could be produced also by the lungs.

This research can also explain the observa-

TABLE II. Fluid Production from Lung of Guinea Pig Fetuses.

Fetal wt, g	Period of fluid production, hr	Vol of fluid produced	Flow, ml/kg/hr
100	4.0	1.10	2.8
94	2.5	1.00	4.3
90	1.0	.12	1.3
86	1.0	.12	1.4
120	1.25	1.00	6.7
100	.5	.15	3.0

* These experiments were performed in the Dept. of Physiology of Yale Medical School.

tion of Reynolds(6), that 30 ml of liquid flowed from the nasopharyngeal oral cavity of a goat fetus in a 2 hour period, and confirms the observation and the hypothesis of Dawes (7).

Inasmuch as no liquid entered the trachea during experiments, the hypothesis that amniotic fluid is absorbed by the lung seems improbable. This hypothesis was proposed by Davis and Potter(8), on the basis that radio-opaque material injected into the amniotic sac was found in the bronchi, but this result can be explained by respiratory movements of the fetus. If amniotic fluid were absorbed in the lung the corpuscles that normally are present in the amniotic fluid would be collected in the lung causing impairment of respiratory function at birth.

The volume of liquid coming out in these experiments represents likely minimal values. If the hypothesis of an ultrafiltration is correct a decrease of hydrostatic pressure in the capillaries would be sufficient to decrease or discontinue the production of fluid. It seems quite probable that such a decrease takes place as a consequence of anesthesia and of surgical shock.

The lung seems to be a source of amniotic fluid, and of particular importance in those animals in which the urethra becomes patent only late in fetal life. In animal in which the urethra becomes patent earlier (*e.g.* man and guinea pig) the kidneys also contribute to production of amniotic fluid. This organ produces a large amount of hypotonic fluid in the last third of fetal life, without waste products found in urine of extrauterine life. The amount of fluid produced by kidneys of the fetus is 6-10 ml/kg/h in goats(9), 0.1-1 ml/kg/h in guinea pigs(10), and about 1 ml/kg/h in rats(11).

Both lungs and kidneys in the guinea pig can therefore produce amniotic fluid at a rate of 1-10 ml/kg/h. This rate of production is about 10-100 times less than the rate of transfer of water to the amniotic fluid found in the guinea pig by Flexner and Gellhorn(12). However, as shown by Paul, *et al.*(13), these results are mainly due to interdiffusion phenomena of water through the permeable barriers that enclose the amniotic cavity and are therefore not the expression of a net transfer or a production of water. This production of fluid is likely small, inasmuch as there is not a continued and large flow of liquid after rupture of the amniotic sac.

It is therefore important to distinguish between interdiffusion and net production of fluid. Interdiffusion is a general phenomenon (less intense in the amniotic sac than in other parts of the organism(14)), resulting from thermic movement of molecules and taking place through every water-permeable membrane. The net production of fluid is caused by ultrafiltration or secretion, and requires energy expenditure and specialized organs. Only the net production of liquid can give rise to a collection of fluid in the organism, and the amniotic fluid is an example of this collection.

Summary. Fluid production by fetal lung of guinea pigs and goats has been demonstrated through cannulation of the fetal trachea in the last part of the pregnancy. This fluid is probably produced by an ultrafiltration process because its physico-chemical features in the last third of pregnancy are very similar to those of the interstitial fluid. It is suggested that the fetal lung is an important source of amniotic fluid during this period of fetal life.

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Biological Activities of Aggregated Gamma Globulin I. Skin Reactive and Complement-Fixing Properties of Heat Denatured Gamma Globulin.* (25116)

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In earlier publications on skin reactivity and complement (C′) fixing potency of soluble antigen-antibody complexes, it was shown that these biological activities are dependent on 2 properties of the complexes, *i.e.*, their composition with respect to antigen/antibody ratio and the species from which the antibody was derived(1,2). When complexes were composed of immune reactants in the molar ratio Ag_2Ab or when the antigen was a simple hapten, the biological activities of skin reactivity and C′-fixation were lacking. Moreover, it was observed that formation of soluble complexes with biological activities was accompanied by increase in levorotation (3). A mechanism for the observed changes in optical rotation was suggested, which postulated that the increase in levorotation might be due to interaction between antibody molecules brought into apposition by antigen. The possibility was also considered that this antibody-antibody interaction might result in induction of skin reactivity and fixation of C′ (3). These considerations were restricted to those complexes containing human or rabbit antibody which manifested the biological activities. It was therefore anticipated that the interaction of human or rabbit antibody (gamma globulin) molecules, induced by reagents other than specific antigen, might also be accompanied by a development of skin reactive and C′-fixing properties. Since it has been shown that the molecular configuration of gamma globulin changes on heating with formation of aggregates, this denatured protein was used to test this possibility. The experiments described below indicate that human gamma globulin aggregated by heat can provoke skin reactions in normal guinea pigs and can also inactivate C′. Aggregated bovine gamma globulin exhibits neither of these properties.

Materials and methods. Gamma globulin. Two preparations of human gamma globulin (HGG) were used, a pooled commercial human Fr. II (Squibb) and a preparation obtained through the courtesy of Dr. W. L. Hughes.‡ The bovine gamma globulin (BGG) was Fr. II (Armour & Co.). In free boundary electrophoresis in barbital buffer (pH 8.6, $\mu = 0.1$), the purified HGG (Hughes) showed a single sharp peak. The BGG contained approximately 5% of a component with mobility of -2.6×10^{-5} cm^2 volt^{-1} sec^{-1}. The tests for skin reactivity of gamma globulin were carried out in guinea pigs in the same manner previously described for testing activity of soluble antigen-antibody complexes(4). The gamma globulin preparations were injected in

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volumes of 0.1 ml into the shaved skin of the back. The animals then received an intravenous injection of Evans Blue and the reactions were estimated from inside of skin 30 minutes later. *Complement fixation test.* Pooled guinea pig serum was used as the source of C′. It was obtained in frozen state from Hyland Laboratories, Los Angeles, Calif. The amount of C′ inactivated by gamma globulin preparations was determined by the method of Mayer *et al.*(5). The diluted test samples were added to 100 $C'H_{50}$ (50% units of C′) in final volume of 10 ml. After 19 hours at 4°C, the residual C′ activity was determined. The number of $C'H_{50}$ inactivated was calculated by subtraction of units left after incubation from the number present in the control containing only buffer and C′. Concentration of each sample for testing was selected so that the number of $C'H_{50}$ inactivated would represent 20 to 80% of hemolytic activity present in the control. $C'F_{50}$, the quantity of each preparation required to fix 50 out of 100 $C'H_{50}$, was calculated according to the method described in(6). C'_3 titrations were carried out by the method of Rapp *et al.* (7). Guinea pig serum was treated with formalin and then reacted with sensitized sheep erythrocytes to yield the intermediate reaction product, $EAC'_{1,4,2}$. These cells served as substrate for estimation of C'_3 in terms of C'_3H_{50}. The quantity of gamma globulin required to inactivate 50 C'_3H_{50} out of 100 units was calculated as described above for determination of $C'F_{50}$.

Results. A. *Skin reactive properties.* The first studies were made with HGG. Aliquots of a 1% solution of HGG (Hughes) in borate buffered saline (pH 8.0) were heated 20 minutes at several temperatures ranging 56°C to 68°C. At temperature higher than 60°C, the preparations were opalescent and at 68°C a precipitate formed. Turbidities of preparations determined at 400 mμ in the Beckman spectrophotometer, model DU, are shown in Table I. Twofold dilutions of these preparations and of unheated HGG were tested for their capacity to enhance capillary permeability with results shown in Table I. It will be seen that HGG heated at 63°C showed augmented skin reactivity at a level of 3 μg N in a volume of 0.1 ml, whereas the unheated HGG did not show this property at a level of 32 μg N.

An attempt was made to purify the skin reactive component in the heated HGG preparation. Two g of Fr. II (Squibb) were dissolved in 100 ml borate buffered saline (pH 8) and centrifuged to remove the insoluble residue. The supernatant was precipitated with sodium sulfate at pH 8. The precipitates obtained at .62 mole/l of sodium sulfate and at 0.62 to 1.18 moles/l were designated Fractions A and B respectively. Both precipitates were dissolved in, and dialyzed against borate buffered saline. A moderate degree of opalescence was observed in Fraction A. Fraction B was entirely clear and showed a single peak ($\mu = -1.2 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}) in free boundary

TABLE I. Effect of Heating on the Skin Reactivity and Complement-Fixing Properties of Human Gamma Globulin.

Temp., 20 min., °C	Optical density*	Min skin reactive dose†	Quantity required to inactivate 50 $C'H_{50}$	
			In buffer‡	In EDTA (0.01 M)
		μg N		
Unheated	.019	>32	890	>>800§
56	.025	16	450	
60	.075	6	28	>800‖
63	.488	3	17	>800‖
68	4.281	>32	470	

* Optical density of solutions containing 1 mg N/ml at 400 mμ with 1 cm light path.

† These values indicate the minimum quantity required to induce a definite skin blueing, avg diameter more than 8 mm.

‡ The term buffer refers to isotonic veronal buffer containing levels of Ca^{++} and Mg^{++} optimal for hemolytic C′ activity(5).

§ No. of $C'H_{50}$ fixed with 800 μg N was negligible as compared with experimental error.

‖ No. of $C'H_{50}$ fixed with 800 μg N were 12 and 15 $C'H_{50}$ respectively.

TABLE II. Skin Reactivity and Complement-Fixing Properties of HGG and of Heated HGG Fractions.*

Treatment	Fraction	Sodium sulfate, mole/l	Optical density	Min skin reactive dose, μg N	Quantity required to inactivate 50 $C'H_{50}$		Quantity required to inactivate 50 C'_3H_{50}†	
					In buffer	In EDTA	37°C	0°C
					μg N			
Unheated	A	.62	.072	4.0	23	1000	400	>>800
	B	1.18	.019	64.0	1250	>>800§	>>800§	"
Heated	B_h		.358	2.0	6.9	1200	87	"
	Fr. 1	.36	.874	.5	2.8	830	39	"
	2	.62	.256	1.0	4.3	1000	63	"
	3	.81	.024	32	1140			
	4	1.18	.028	32	>800‡			

* See legend for Table I for description of terms.

† These values were obtained using same $EAC'_{1,4,2}$ cells at same time. No. of C'_3H_{50} units left in control tube was approximately 100.

‡ 800 μg N inactivated 13 $C'H_{50}$.

§ Inactivation of C′ or C'_3 activity with 800 μg N was negligible as compared with experimental error.

electrophoresis in barbital buffer of pH 8.6 and ionic strength of 0.1. The protein concentration of this fraction was adjusted to 2% and heated at 63°C for 20 minutes at which time the solution became markedly opalescent. This heated HGG (Fr. B_h) was fractionated again with sodium sulfate according to method described by Christian(8) to yield 4 different fractions. These were designated Fr. 1, Fr. 2, Fr. 3, and Fr. 4, and had been precipitated successively at 0.36 mole/l, 0.36 to 0.62 mole/l, 0.62 to 0.81 mole/l, and 0.81 to 1.18 moles/l of sodium sulfate. As indicated by optical density measurements in Table II, only the first 2 fractions of the heated HGG were opalescent. The data in Table II also show that Fraction A had skin reactive properties, but Fraction B did not. The increase of skin reactivity resulting from heating was also confirmed. The denatured protein in Fr. B_h, presumably responsible for its skin reactivity, was concentrated in Fr. 1 and 2 on precipitation with sodium sulfate. It may be noted that as little as 0.5 to 1.0 μg N of these fractions gave definite skin reactions.

Since bovine antisera generally lack sensitizing activities for guinea pigs(9), it was of interest to study the skin reactive properties of heated BGG. Aliquots of a 1% solution of BGG, at pH 8.0, were heated 20 minutes at 56°C, 63°C, and 65°C. The solution heated at 56°C was clear whereas the other 2 were opalescent with a turbidity comparable to analogous HGG preparation. These BGG preparations, however, failed to produce skin reactions with as much as 32 μ N.

B. *Complement-fixing properties.* Inactivation of C′ by HGG preparations described in the previous section was studied. Appropriate dilutions of the preparations were added to 100 $C'H_{50}$ units of C′ and incubated at 4°C for 19 hours. The C′-fixing potencies were compared in terms of $C'F_{50}$, *i.e.*, the quantity (μg N) of each sample required to fix 50 out of 100 $C'H_{50}$. The results obtained are shown in Tables I and II. It may be seen that the HGG heated at 63°C was much more active than the unheated control both with respect to C′ inactivation and skin permeability enhancement. The parallelism between skin reactivity and C′-fixing potency was also confirmed with the several fractions of heated HGG (Table II). It is perhaps worthy of special note that Fractions 1 and 2 were highly potent in C′-fixation and also showed highest skin reactivity.

In an attempt to compare the mechanism of C′ inactivation by heated HGG and by antigen-antibody complexes, the effect of a chelating agent was explored. It has been amply demonstrated that EDTA deprives the reaction system of the divalent cations required for interaction of C'_1, C'_4, and C'_2 with antigen-antibody complexes(10). In the present experiments, Na_3HEDTA, in final concentration of 0.01 *M*, was added to the reaction mix-

tures containing 100 $C'H_{50}$ and 1 ml of 0.5% HGG preparations in total volume of 4 ml. After incubation at 4°C for 19 hours, 0.4 ml of 0.1 *M* $CaCl_2$ was added to each mixture which was then diluted 50 times with barbital buffer, containing optimal concentrations of Ca^{++} and Mg^{++}, and the residual C′ was titrated. The data in Tables I and II indicate that inactivation of C′ by heated HGG is inhibited in presence of EDTA. It has been shown that inactivation of C'_3 by antigen-antibody reactions is dependent on temperature of incubation(11). These findings were duplicated in experiments with heated HGG. One ml volumes of appropriate dilutions of unheated HGG, of heated HGG, and of the several fractions were mixed with 100 $C'H_{50}$ of C′ in total volume of 10 ml. After incubation for 1 hour at 0°C or 37°C, the residual C'_3 activity in each reaction mixture was titrated with results shown in Table II. There was significant inactivation of C'_3 by Fraction A of unheated HGG, heated HGG (Fr. B_h) and its Fractions 1 and 2, in contrast to Fraction B of unheated HGG. It was also observed that inactivation of C'_3 is markedly dependent on temperature. At 0°C, no significant loss of C'_3 was observed with any of the preparations. The behaviour of heated HGG and of antigen-antibody complexes is therefore similar in this regard.

The C′-fixing potency of BGG preparations was also studied. One ml volumes of 0.5% solutions of either unheated or heated BGG (20 minutes at 56°C, 63°C, and 65°C) were incubated with C′ and the residual C′ was titrated. The anticomplementary effect of BGG was extremely low and not increased by heating.

Discussion. The present experiments clearly show that heat denatured preparations of HGG can enhance permeability of minute vessels in the guinea pig skin and fix C′. On fractionation of heated HGG with Na_2SO_4, these activities are concentrated in Fr. 1 and 2 (Table II) which are opalescent. The proteins in these fractions seem to be aggregated as judged in terms of decreased solubility and increased turbidity. With respect to unheated HGG, Fraction A obtained by precipitation with sodium sulfate (0.62 mole/l) also possesses both the biological activities under study. These experiments and those of Christian(5) indicate that salt fractionation provides a method for concentrating the aggregated gamma globulins which can induce the immediate type of skin reaction. It may also be noted that aggregated HGG (Fr. 1) yielded a definite skin reaction at a level of 0.5 μg N. This value is comparable to the minimum skin reactive dose of soluble antigen-antibody complexes. It was found previously(2) that the approximate amount of antibody in the minimum skin reactive dose of active complexes was 0.2 to 0.8 μg N. It is therefore evident that aggregated HGG and soluble antigen-antibody complexes are quantitatively comparable with respect to their skin reactive properties.

The data in Tables I and II show that skin reactivity and C′-fixing properties of heated HGG parallel each other, in agreement with the parallelism observed in studies with soluble complexes(2). The lack of skin reactivity shown by heated BGG is in accord with observations that activity of soluble complexes depends on properties of the antibody(2) and that bovine antibody is incapable of sensitizing guinea pigs(9). If the aggregated gamma globulin and the soluble complexes operate through similar mechanism, it would follow that the fixation of the altered gamma globulin to the tissues should be one of the necessary pre-requisites for the skin reaction(12). In this sense, inactivity of aggregated BGG might be due to low affinity for guinea pig tissues as well as its deficiency in C′-fixing capacity (*cf.* 12).

With respect to possible relationship of the present findings to C′-fixation, Davis, *et al.* (13) reported that HGG was anticomplementary and Olhagen(14) showed that aggregation of these proteins was responsible for this effect. In our study it was found that 2.8 μg N of Fraction 1 of heated HGG inactivated 50 out of 100 $C'H_{50}$. This value is of the same order of magnitude as that reported by Wallace, *et al.*(6) who found that the weight of rabbit anti-BSA required to fix 50 units out of 100 units in the cold with optimal amount of antigen varied from 1 to 3.94 μg N. Osler(15) has recently presented data for

human antibody showing that 2.36 μg N of human Wassermann antibody fixed 35 to 38 out of 50 $C'H_{50}$ of C′ with optimal amounts of cardiolipin antigen, although incubation time was only 90 minutes at 37°C in this experiment. From these comparisons it seems that the aggregated HGG and antigen-antibody complexes have C′-fixing potencies which are of the same order of magnitude. Inactivation of C′ by aggregated HGG requires divalent cation(s) as is also the case in fixation of C′ by antigen-antibody reactions(15). Inactivation of C'_3 and its dependence on temperature are also in agreement with the findings for C′-fixation by antigen-antibody complexes(10,11). The present results, therefore, suggest that the mechanism of C′ inactivation by heated HGG and by antigen-antibody complexes might be similar. Lack of C′-fixing properties shown by heated BGG is in agreement with the fact that only a small proportion of cattle develop C′-fixing antibody (16,17).

An explanation of the mechanism involved in skin reactions and C′ inactivation by aggregated HGG must await further studies of the aggregated protein as obtained by methods other than heat denaturation. However, comparable capacities of aggregated HGG and of antigen-antibody complexes with respect to skin reactive and C′-fixing properties suggest a similarity in mechanism. In our previous studies, evidence was presented that certain soluble antigen-antibody complexes show changes in molecular configuration of antibody and also exhibit skin reactive properties (3). It was suggested that this molecular change might be due to interaction between antibody molecules, brought into close proximity by antigen(3). The present experiments indicate that aggregated HGG, without participation of any antigen, can produce a toxic effect comparable to soluble complexes. This finding supports our hypothesis that the skin reactivity shown by the soluble complexes is due to antibody-antibody interaction and/or the consequent change in their configuration. In terms of this hypothesis, skin reactions might be produced as a result of combination of such altered antibody molecules to the tissues(3,12).

With respect to the mechanism of C′-fixation by antigen-antibody reactions, Heidelberger, *et al.*(18) postulated that components of C′ enter into easily dissociable compounds with antibody molecules in the absence of antigen and when antigen is introduced, the molecules of C′ become firmly bound in immune aggregates. Hill and Osler (19), in support of this hypothesis, concluded that C′-fixation increases with aggregating capacity of antibody. The present data may be considered to extend Heidelberger's idea through the demonstration that aggregated HGG by itself—without participation of any antigen—is entirely comparable to antigen-antibody complexes in inactivation of C′. If the mechanism of C′ inactivation by aggregated HGG is actually the same as the inactivation by antigen-antibody complexes, a possible mechanism of C′-fixation may be suggested. Thus, interaction between antibody molecules brought into apposition by antigen, with resulting change in their configuration, might be responsible for fixation of C′ by antigen-antibody reactions. This idea is in accord with the fact that neither the Ag_2Ab complex nor the simple hapten-antibody complex fix C′(2). Since aggregated BGG does not fix C′, the interaction between gamma globulin (antibody) molecules is probably not the only determinant of C′ fixation. The dependence of C′-fixing properties of antigen-antibody complexes on the species from which the antibody is derived suggests that the properties of antibody molecule may be another important determinant of C′ fixation. The hypothesis outlined above would therefore be restricted to those complexes containing antibody of the rabbit or human type.

Finally, our findings are also in accord with the hypothesis that C′ may be an essential reagent in the mediation of allergic reactions of the immediate type(11,20). The present data merely provide another set of correlated observations in this regard and suggest that a similar change of gamma globulin (antibody) might be responsible for both the skin reactivity and C′-fixing properties. In any event, these and previous observations(3) suggest that the interaction between gamma globulin (antibody) molecules might play an impor-

tant role in many biological phenomena which follow antigen-antibody combination, such as C′-fixation, skin reaction with soluble complexes, as well as anaphylactic reactions. It is of interest that aggregated gamma globulins give allergic-like responses and this suggests the possibility that some allergic dieases might be induced by molecular changes of the gamma globulin *in vivo*.

Summary. 1. Aggregated human gamma globulin, obtained by heating, gave immediate skin reactions in normal guinea pigs and inactivated complement, whereas aggregated bovine gamma globulin did not have either activity. 2. Aggregated human gamma globulin and antigen-antibody complexes are comparable on weight basis with respect to skin reactivity and complement inactivation. 3. Divalent cation(s) are required for inactivation of complement by aggregated HGG. Inactivation of C′3 by aggregated HGG is markedly dependent upon temperature. 4. A tentative hypothesis concerning the mechanism of complement fixation by antigen-antibody reaction is presented.

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Effect of Sodium Chloride Feeding on Adrenocortical Hormone Secretion of Salt Deprived Rats.* (25117)

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Sodium deprivation in rats results in increased secretion of aldosterone but total adrenocortical hormone production is decreased because of diminished synthesis of corticosterone and other steroids(1). These changes in hormone secretion take place gradually after withdrawal of sodium from the diet and require several weeks to become fully manifest. Alterations in adrenal steroid secretion induced by sodium restriction are reversible but it is not known how long it takes for cortical hormone production to become normal once adequate sodium intake is resumed. The purpose of this investigation was,

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TABLE I. Body Weights, Adrenal Weights, and Adrenocortical Hormone Secretion of Various Groups (Mean Values ± Stand. Error of Mean).

Group	Body wt (g)	Adrenal wt (mg/100 g body wt)	Adrenal steroid secretion (μg/100 mg adrenal wt)
Sodium deficient rats (16)*	120.1 ± 6.3	18.85 ± .93	55.7 ± 4.0
1 day salt replacement (8)	127.6 ± 8.0	17.37 ± .97	76.1 ± 7.4
2 " " " (8)	146.3 ± 8.7	15.55 ± .78	101.7 ± 9.6
Pair fed controls (16)	180.9 ± 2.3	13.92 ± .68	95.6 ± 6.6

* No. of animals in each group.

therefore, to determine the effect of salt replacement on secretion of cortical hormones by the adrenals of salt depleted rats.

Methods and procedure. Thirty-two male Wistar rats, weighing 50-60 g, were fed a sodium deficient, synthetic diet *ad lib.*(2). Sixteen control rats received the same diet to which was added 0.24% sodium (as sodium chloride) and were pair-fed with the deficient group. After the experiment was in progress 1 month, 16 rats were randomly selected from deficient group and fed the salt-containing (control) diet *ad libitum*. Eight of these animals consumed control diet for 1 day and the other 8 for 2 days before being sacrificed. Rats remaining in deficient group were maintained on the sodium-free regimen for 1 or 2 more days and were then sacrificed along with control animals. Animals were killed by decapitation and adrenals dissected free of surrounding tissue, weighed and bisected. Both adrenals from each rat were placed in 2 ml of Krebs-Ringer-phosphate solution, pH 7.4, which contained 200 mg % glucose. One unit of ACTH was added and the mixture incubated aerobically for 2 hours at 37°C in a Dubnoff Metabolic Incubator. On completion of incubation steroid hormones secreted into medium were recovered and determined by a method which has been previously described (3). This procedure utilizes a spectrophotometric technic which measures steroid compounds possessing an alpha, beta unsaturated ketone structure in ring A. After adrenal steroid secretion of each animal was determined, the solution containing the hormones was combined with that from other animals in the group and individual steroids separated by paper chromatography(4).

Results. (Table I). Rats fed the sodium deficient diet gained weight slowly but the final mean body weight of this group was significantly less than that of pair fed controls ($P = <.001$) or the group refed salt for 2 days, $P = <.025$). Average body weights of deficient rats and those refed salt 1 day differed only slightly.

Adrenal hypertrophy occurred in salt deprived rats as demonstrated by an increased adrenal weight-body weight ratio (AW/BW) when compared to that of pair-fed controls, ($P = <.001$). The AW/BW of rats given salt replacement for 1 day was less than that of the deficient group but this difference was not large enough to be statistically significant. Two days of salt refeeding resulted in a further decrease in adrenal weight, however, so that the difference in AW/BW between this group and deficient rats was significant, $P = <.05$.

Hormone secretion by adrenals of sodium depleted rats was approximately one-half that of control animals, the difference being highly significant, $P = <.001$. After 1 day of salt replacement there was an increase in steroid secretion so that difference in hormone production by this group and deficient rats was significant, $P = <.05$. A further increase in adrenal hormone secretion occurred in animals given salt refeeding for 2 days so that the value for this group was about the same as that of control rats. Difference in hormone production by the group refed salt for 2 days and deficient group was significant, $P = <.001$.

Previous investigation has shown that isolated adrenals of normal rats secrete aldosterone, corticosterone and other hormones with corticosterone being produced in largest amount(5,6,7). Adrenals of sodium depleted rats secrete relatively large amounts of aldosterone but elaboration of other hormones vir-

tually ceases if salt restriction is prolonged (1). In this study chromatographic separation of steroids secreted by adrenals of control rats showed a normal pattern. Steroid production by adrenals of rats depleted of sodium for 1 month consisted almost entirely of aldosterone and no corticosterone could be detected. Chromatographic analysis of adrenocortical hormone secretion of rats fed a salt-containing diet for 1 day after being deprived of sodium for 1 month revealed an increased amount of aldosterone, although the quantity was less than that produced by depleted animals not subjected to salt replacement. Furthermore, a small amount of corticosterone was secreted by adrenals of these rats whereas adrenals of deficient animals did not produce this hormone. The pattern of adrenal steroid secretion after 2 days of salt replacement was essentially normal, although a slight increase in aldosterone was still evident.

Discussion. Sodium deficiency induced in rats results in alterations of adrenocortical structure and function. Hypertrophy of the zona glomerulosa and increased aldosterone secretion occur and there is secondary atrophy of the zona fasciculata with reduced synthesis of corticosterone(1,2,8). These changes gradually become more marked as salt deprivation is continued and body sodium content declines. Studies of the effect of salt replacement on the adrenal cortex of sodium depleted rats have shown that structural abnormalities are partially corrected after 24 hours and are almost completely reversed within 48 hours(9). Reappearance of a normal histological pattern after salt refeeding was considered to indicate that cortical hormone secretion had returned to the original level although steroid determinations were not carried out.

In our experiment it was demonstrated that resumption of salt feeding after a period of sodium restriction resulted in a rapid increase in total cortical hormone secretion so that within 2 days normal levels were attained. Furthermore, as body sodium stores were replenished there was a decline in production of aldosterone and synthesis of corticosterone, which had virtually ceased, returned to normal. These observations show that abnormalities of adrenal steroid secretion induced by salt depletion can be quickly corrected when sodium concentration approaches physiological levels. Changes in sodium content undoubtedly affect cortical hormone production by altering activity of certain enzyme systems although the mechanism by which these reactions are influenced is not understood. Regardless of mechanism of control it is apparent that body sodium content is an important factor affecting hormone secretion by the adrenal cortex.

Summary. Effect of salt replacement on secretion of adrenocortical hormones by rats which had been deprived of sodium for 1 month was investigated. Results demonstrated that total cortical hormone production, which had been depressed, increased to equal that of control animals; that elevated aldosterone synthesis declined to previous levels; and that cortisterone secretion, which had been undetectable, increased rapidly. Thus, within 48 hours after resumption of salt feeding secretion of adrenocortical hormones returned to normal.

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A Tissue Culture Color Test for Measuring Influenza Virus and Antibody.* (25118)

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The effect of poliovirus on metabolic activity of cultures of human embryonic tissue was observed by Enders, *et al.*(1) who found that infected cultures were less active than uninoculated controls in regard to acid formation. Later, they observed characteristic cytopathology in roller tube cultures and adopted this method for determination of the presence of virus since inhibition of acid formation was not always consistent(2). Salk, Youngner and Ward(3) developed a colorimetric test for assay of poliovirus and antibody *in vitro* using color change of phenol red as an indication of presence of virus or antibody. The present report describes a similar color test for titration of influenza virus and antibody. This test offers certain advantages over the more cumbersome egg technics currently in use.

Materials and methods. Media. Lactalbumin medium with 5% bovine serum was used for growing cells in 32 ounce prescription bottles and medium 199 without serum was employed for virus titrations and neutralization tests. Optimum color contrast could be obtained after 7 days if undiluted 199 was used with 2.5 ml of 5% sodium bicarbonate solution/100 ml of medium. Penicillin and streptomycin were added to concentration of 200 units and 200 μg/ml, respectively. Cell suspensions were prepared from kidneys of normal rhesus monkeys by methods previously described(4). Secondary cell suspension was prepared from bottle cultures by dispersing cells with 1:5000 sodium versenate and resuspending them in medium 199 without serum to concentration of 280,000 cells/ml. To each Wassermann tube was added 0.25 ml of cell suspension, final volume brought to 0.75 ml with medium 199 and the fluids overlaid with 0.5 ml of heavy mineral oil. *Virus pools* were prepared in monkey kidney roller tube cultures and stored at 4°C for a period not exceeding 2 weeks when they were replaced with fresh pools. *Immune sera.* Emulsified mineral oil adjuvant vaccines were prepared using Arlacel A as emulsifying agent. Antigens consisted of monkey kidney tissue culture pools or allantoic fluid pools of types A, A′, B, B′, and Asian influenza viruses. Normal rhesus monkeys or White Leghorn chickens were inoculated with adjuvant vaccines and were exsanguinated 6-8 weeks later following several additional aqueous vaccine inoculations. All sera were heated at 56°C for 30 minutes to destroy heat-labile non-specific inhibitors. Monkey sera were also treated with 8 mg trypsin (Difco 1:250)/ml of serum and chicken sera with 2 volumes 0.01 M potassium meta-periodate/volume of serum. *Hemagglutination and hemagglutination-inhibition* tests were performed according to standard methods(5).

Results. Virus titrations. Serial 10-fold dilutions of virus were made in medium 199 without serum and titrations performed as described elsewhere(3). Eight to 10 replicate tubes/dilution were used. Results of a typical titration are presented in Table I. In addition, virus titers were compared by color test and roller tube methods and, as observed in Table II, the mean color test titer was 0.56 logs lower than titers obtained in roller tubes.

Antibody titrations. Serial 2-fold dilutions of serum were made and to each dilution an equal volume of virus suspension containing about 400 $TCID_{50}$ of virus/ml was added. The mixtures were incubated at room temper-

TABLE I. Color Test Titration of Influenza Virus Infectivity Strain 575 MK-11 (Type A′).

Virus dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Score*	8/8	8/8	8/8	8/8	6/8	2/8	0/8

Virus titer: $10^{5.5}$ ID_{50}/0.5 ml

* Numerator = No. of tubes infected (medium red). Denominator = No. of tubes inoculated.

* This investigation was carried out under sponsorship of Commission on Influenza of Armed Forces Epidemiological Board, and supported by Office of Surgeon General, Dept. of Army.

TABLE II. Comparison of Roller Tube and Color Test Infectivity Titrations of Influenza Viruses.

Virus	Type	Neg. log of ID_{50}/0.5 ml: Roller tubes	Color test	Diff. (RT – CT)
PR8 MK 12	A	6.0	4.8	1.20
" 10	A	4.74	4.11	.63
			4.0	.74
			4.0	.74
			4.5	.24
" 14	A	5.5	4.95	.55
575 MK 11	A′	5.5	5.4	.10
1210 MK 8	B′	5.5	5.0	.50
1210 E 4	B′	7.5	6.28	1.22
Peter MK 6	B′	3.25	3.56	−.31
				$\bar{X}$ = .56 logs

ature for 30 minutes and 0.5 ml of each serum-virus mixture was transferred to 3 or 4 replicate tubes. Each tube then received 0.25 ml of previously standardized cell suspension. Appropriate controls were incorporated as described previously(3). In an occasional uninfected tube, the pH did not fall to 6.6 or 6.8. However, it was observed microscopically that cultures having pH 7.4 or lower rarely displayed cytopathology. These tubes were considered negative (yellow) while tubes with pH higher than 7.4 were considered positive (red). A representative antibody titration is shown in Table III.

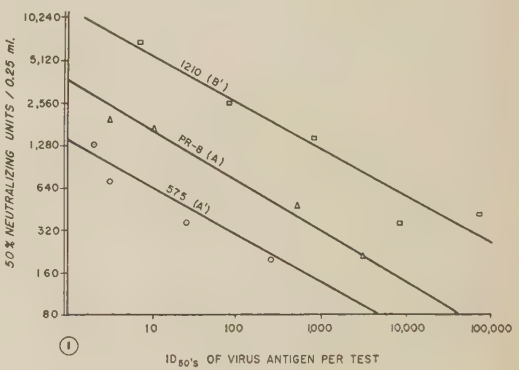
Influence of antigen concentration on antibody titer. Neutralization tests were performed with tissue culture and egg adapted strains in the color test and *in ovo* by varying both serum and virus concentrations. Types A, A′, and B′ were tested and, as shown in Fig. 1, a straight line relation existed: a 2-fold change in antibody titer resulted from each 10-fold change in antigen concentration. Slopes of neutralization curves did not differ significantly with virus type or when neutralized mixtures were assayed in the color test or *in ovo*. The curves in Fig. 1 are from combined data of egg and color test assays. Clarke and Tyrrell(6) assayed neutralized mixtures of influenza virus and antibody in monkey kidney roller tube cultures and in embryonated eggs and found the slope for the neutralization curve in cell culture was essentially the same as reported here. In contrast, a less steep slope was reported using *in ovo* assays than we observed.

TABLE III. Color Test Titration of Influenza Antibody Content of Immune Monkey Sera.

Antigen: Strain 575 (A′), 100 ID_{50}/tube

Serum dilution	Immune sera: Anti-A′	Anti-A	Anti-B
1:20	0/3*	3/3	3/3
1:40	"	"	"
1:80	"	"	"
1:160	"	"	"
1:320	"	"	"
1:640	3/3	"	"
1:1280	"	"	"
1:2560	"	"	"
50% neutralizing units/0.25 ml serum	480	0	0

* Numerator = No. of tubes infected (medium red). Denominator = No. of tubes inoculated.



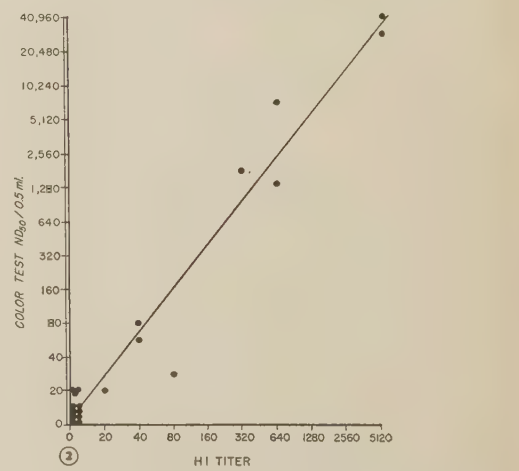


FIG. 1. The influence of amount of antigen on neutralizing antibody titers of influenza antisera.
FIG. 2. Correlation of color test neutralization and HI titers of periodate treated hyperimmune chicken sera.

Determination of antibody in immune

TABLE IV. Specificity of Hyperimmune Monkey Sera Tested with Homologous and Heterologous Virus Antigens.

Sera	Virus strain						
Antigen (immunization)	Swine	PR8 A	575 A′	Cup A′	FM-1 A′	Wright A′	1210 B′
Pre-vaccination	0	0	0	0	0	0	0
Post-vaccination							
Swine	60	0	0	0	0	0	0
PR8 A	0	90	0	0	10	10	0
575 A′	0	15	960	1920	1920	1920	0
"	0	10	60	30	480	30	0
"	0	15	60	30	480	30	0
1210 B′	0	0	0	0	10	0	1920
"	0	0	0	0	0	0	960
"	0	0	0	0	0	0	1920

All sera were heated at 56° for 30 min. and treated with 8 mg trypsin/ml serum. Serum antibody titers of all pre-vaccination sera were less than 1:10, lowest dilution tested. Values shown are 50% neutralizing doses/0.5 ml.

TABLE V. Color Test Titrations of Human Acute and Convalescent Sera Using Different Types of Influenza Virus as Antigens.

		Virus strain						
Patient	Bleeding	Swine	PR8 A	575 A′	Cup A′	FM-1 A′	Wright A′	1210 B′
Els	Acute	0	260	0	0	60	60	160
"	Conval.*	0	480	0	120	480	480	240
Sch	A	0	260	0	0	80	480	960
"	C	80	480	0	120	480	960	960
Swt	A	120	0	0	0	0	40	240
"	C	480	120	0	120	480	750	240
Wei	A	0	120	0	0	80	60	0
"	C	0	480	60	480	480	2980	0
Har	A	0	60	0	0	0	0	60
"	C	0	480	0	0	0	40	60

* Convalescent.

Sera were heated at 56° for 30 min. and treated with 8 mg trypsin/ml serum. Lowest dilution tested was 1:40 and values shown are 50% neutralizing doses/0.5 ml.

monkey sera. Cross neutralizations were performed with immune monkey sera using types A, A′, B′ and Swine as virus antigens. All pre-vaccination sera were free of neutralizing activity (Table IV). With immune sera, significant levels of clearly type specific antibody were obtained with the exception of some inhibition of PR8 virus with the A′ sera.

Determination of antibody in human sera. Acute and convalescent sera† from patients with clinically diagnosed influenza were tested with 4 types of virus to detect any significant increases in antibody level. Convalescent sera were obtained 2 weeks after the acute phase bleeding. Four-fold or greater rises in antibody titers were found with A′ virus antigens and especially with strains FM-1 and Wright (Table V). The response of Patient Har was significant with type A virus. Since the initial dilution of serum in these titrations was 1:40, the apparent increases of some titers may not be significant.

Hemagglutination-inhibition and color test antibody determinations with immune chicken sera. Cross neutralizations were performed using 5 type antigens and 4 periodate treated immune sera. Both HI and color tests were done with the same virus pools and the same series of serum dilutions. Antibody levels found with both methods were type specific and were increased with homologous type antigens (Table VI). The mean antibody titer by the color test method using homologous

† Kindly supplied by Elva Minuse, Virus Laboratory, School of Public Health, University of Michigan.

TABLE VI. Comparison of Color Test and HI Titrations of Hyperimmune Chicken Sera Using Homologous Influenza Virus Antigens.

	Antisera‡							
	PR8		PR301		Formosa		GL 1739	
Virus	CT*	HI†	CT	HI	CT	HI	CT	HI
PR8	30,700	5120	20	0	0	0	0	0
PR301	30	80	40,960	5120	60	40	20	20
Formosa	0	0	0	0	7680	640	0	0
GL 1739	80	40	20	0	20	0	1500	640
MB	0	0	0	0	0	0	1920	320

* No. of ND_{50}/0.5 ml. † Reciprocal of highest dilution showing inhibition. ‡ Heated at 56° for 30 min., treated with 0.01 M potassium periodate.

viruses and sera was 16,552 50% neutralization doses/0.5 ml while mean heterologous titer was 16 ND_{50}/0.5 ml. The mean homologous HI titer was 2368 compared to 12 for the heterologous titer. A plot of the titers is shown in Fig. 2 with regression line determined by method of least squares. The significance of linearity of regression was tested by means of analysis of variance. It was concluded that at the 0.10% confidence level there is a significant linear relationship over and above any curvilinear factors.

Conclusions. As with poliovirus, color test titrations of influenza virus and antibody represent an efficient, economic and accurate research tool which could be of great value in large scale epidemiological surveys and in detailed antigenic analyses of influenza virus strains. Economy of method can be demonstrated for about 18,000 Wassermann tube cultures, using secondary cells, can be prepared from kidneys of one 5 lb rhesus monkey. The methods currently employed for quantitative neutralization of influenza viruses are often time-consuming and cumbersome whereas the color test is unique in its simplicity and adaptability. The data presented also demonstrate high degree of serum specificity associated with this method and indicate that sensitivity of color test for detection of unneutralized virus is comparable to that observed *in ovo*.

Summary. 1. A tissue culture color test has been developed for titration of influenza virus and antibody using secondary monkey kidney cell cultures. 2. Virus titrations with the color test are comparable in accuracy with those done in roller tube cultures, mean titer being 0.56 logs lower in the color test. 3. Antibody content of immune sera (animal or human) can readily be determined and slopes of neutralization curves determined by color test are comparable to those found *in ovo*. 4. Color test neutralization titers and HI titers of the same sera show a significant association.

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Linoleic and Linolenic Acids: Their Oxidation by Normal and Diabetic Rats.* (25119)

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The metabolic behavior of saturated and unsaturated fatty acids differs in many important aspects. The β-fatty acids of lecithins isolated from various animals have been shown to be predominantly saturated; those in the α position, mainly unsaturated(1). The fatty acids commonly found in ester linkage with plasma cholesterol are chiefly unsaturated(2-4). Certain of the polyunsaturated fatty acids either are not synthesized by the rat, or are synthesized to a limited extent only (5). Their absence from the diet of young rats may result in a variety of derangements which can be alleviated by addition to diets of small amounts of polyunsaturated fatty acid arachidonic, or its metabolic precursor, linoleic acid. Linolenic acid also relieves deficiency signs, but it is reported to be less effective than the other 2 fatty acids(6). Recently it has been claimed that the 2 types of fatty acids differ in their effects on serum cholesterol in humans, with vegetable oils rich in unsaturated fatty acids lowering serum cholesterol when substituted for the more saturated fatty acids of the diet(7-10). The oxidation of palmitic acid is spared in the intact normal rat by carbohydrate feeding and in the diabetic rat by insulin administrations(11-14). In view of the difference in metabolic behavior of saturated and polyunsaturated fatty acids, and particularly in view of the body's limited (or lack of) capacity to synthesize linoleic acid, it seemed desirable to determine whether catabolism of the 2 principal, naturally-occurring, 18-carbon polyunsaturated fatty acids, linoleic and linolenic, is also controlled by carbohydrate utilization. The effect of insulin upon their utilization in the diabetic rat was also studied because of the interesting observation of Peifer and Holman (15) that depletion of essential fatty acids occurs more rapidly in the diabetic than in the normal rat when they are fed a diet deficient in these fatty acids.

Methods. C^{14}-labeled fatty acids. Linoleic and linolenic acids randomly labeled with C^{14} were obtained as the methyl esters from Dr. Herman Schlenk of the Hormel Institute, Univ. of Minnesota, Austin, and we are greatly indebted to him for a generous supply of both acids. Their photosynthetic preparation and establishment of their purity have been described by Mangold and Schlenk (16). The fatty acids were injected in the form of unesterified fatty acid bound to albumin. They were converted to this form by the following procedure: The methyl esters were saponified with sodium ethoxide at 60° for one hour, the mixture was acidified, and fatty acids were extracted with petroleum ether. The volume of the solvent containing the fatty acids was reduced to a few ml, and the acids were converted to the sodium salts. A few drops of 0.9% saline were added, and volatile solvents were removed in a stream of nitrogen. The sodium salts of the fatty acids were then mixed at 37° with 0.9% saline and rat plasma so as to yield a mixture containing 9 parts plasma to 1 part saline. Plasma proteins were separated by paper electrophoresis in sodium barbital buffer at pH 6, stained with bromphenol blue, and scanned for C^{14}. The albumin fraction was the only one labeled with C^{14}. In one experiment the saline solution of the sodium salt of the fatty acid was divided into 2 parts, one of which was converted to the albumin complex with defatted human albumin, the other, with rat plasma. (The results obtained with these 2 preparations were indistinguishable.) Each of the rats received intravenously 1 ml of the preparation, which contained 0.20 mg of the labeled fatty acid. *Exp. 1.* Long-Evans, normal male rats weighing about 200 g were used. For a few days before the start of the experiment they were fed, by stomach tube, a fluid diet high in carbohydrate, as described elsewhere

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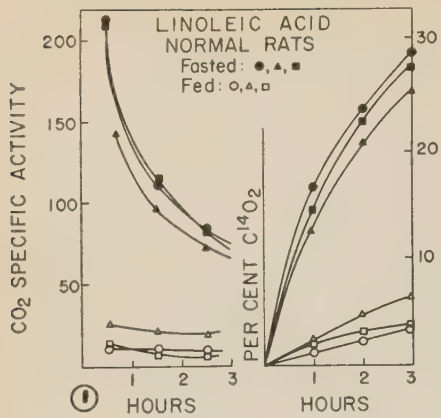

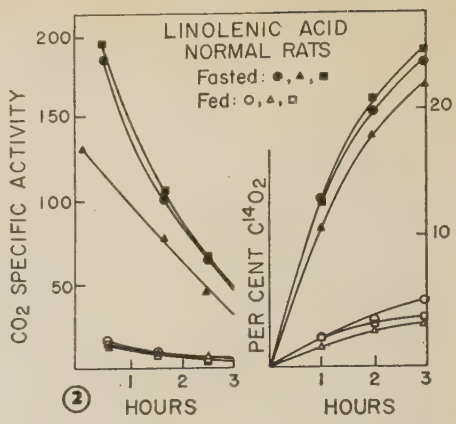

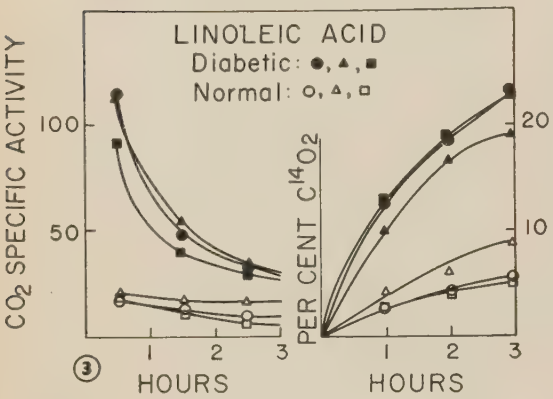

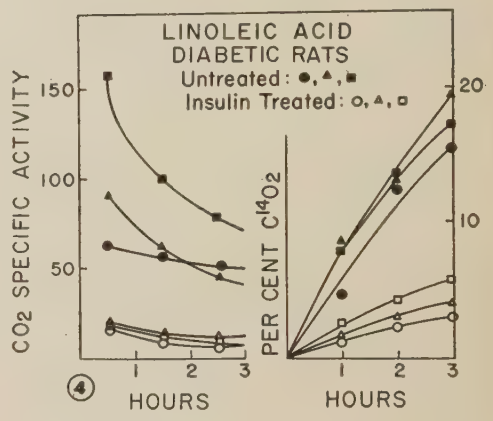

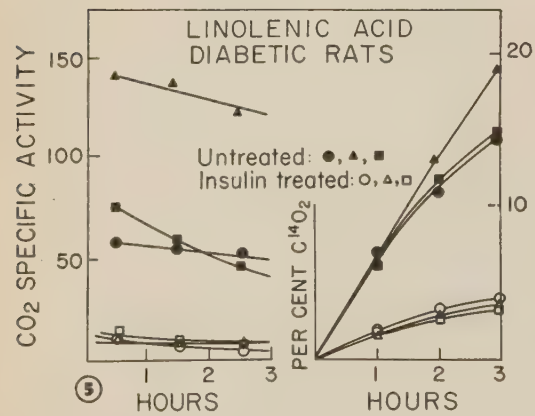

FIG. 1. Cumulative (right) and specific activity values for expired CO_2 obtained with normal rats inj. intrav. with linoleic acid randomly labeled with C^{14}.

FIG. 2. Cumulative and specific activity values for expired CO_2 obtained with normal rats inj. intrav. with linolenic acid randomly labeled with C^{14}.

FIG. 3. Comparison of oxidation of linoleic acid, randomly labeled with C^{14}, by normal and diabetic fed rats.

FIG. 4. Effect of insulin on oxidation of C^{14}-randomly labeled linoleic acid by the diabetic rat.

FIG. 5. Effect of insulin on oxidation of C^{14}-randomly labeled linolenic acid by the diabetic rat.

(11). The fasted rats were fed for the last time 24 hours before start of experiment. The fed rats received their last feeding, 12 ml of a 35% glucose solution, 30 minutes before labeled fatty acids were injected. *Exp. 2.* Diabetes was induced in rats by an intravenous injection of alloxan monohydrate (40 mg/kg of body weight) at least 3 weeks before the rats were used. Daily records of food consumption and urine volume were kept. The rats selected had fasting (6 hours) blood sugar values in excess of 300 mg %, measured a few days before the experiment. Both normal and diabetic rats used in this experiment were fed

TABLE I. Oxidation of Linoleic and Linolenic Acids by Normal, Diabetic, and Insulin-Treated Diabetic Rats.

C^{14}-fatty acid inj.	Hr	Specific activities of CO_2: Norm. fasted* / Norm. fed	Diab. fed† / Norm. fed	Untreat. diab.‡ / Insulin-treat. diab.	Hr	Cumulative % $C^{14}O_2$: Norm. fasted* / Norm. fed	Diab. fed† / Norm. fed	Untreat. diab.‡ / Insulin-treat. diab.
Linoleic	0-1	7.6	6.2	6.0	0-1	5.7	3.4	4.1
	1-2	11.6	4.4	6.1	0-2	9.8	4.3	3.9
	2-3	7.5	3.0	6.4	0-3	5.4	4.4	4.3
Linolenic	0-1	7.9		3.9	0-1	6.0		3.1
	1-2	6.3		4.7	0-2	5.7		3.7
	2-3	6.4		5.3	0-3	5.4		4.1

* Avg of 3 fasted and 3 fed rats (Exp. 1). † Avg of 3 diabetic and 3 normal rats (Exp. 2).
‡ Avg of 3 untreated and 3 insulin-treated diabetic rats (Exp. 3).

ad libitum an adequate stock diet (Diablo abration) until 1 hour before start of experiment; at that time both groups of rats received 6 ml of a 35% glucose solution by stomach tube. *Exp. 3.* Only diabetic rats were used in this experiment, and they were fed, *ad libitum,* a synthetic diet containing 60% glucose(17) for 3 days before start of experiment. The insulin-treated rats received protamine zinc insulin subcutaneously (2 units per 100 g body weight), first at 12 hours and again at 1 hour before labeled fatty acids were injected. *Collection and analysis of expired CO_2.* Immediately after injection of labeled fatty acids, the rats were placed in glass metabolism cages, and the expired air was drawn by reduced pressure into glass columns containing 5N sodium hydroxide. The procedures used for determination of the C^{14} content of expired CO_2 have been described (18).

Results. Figs. 1 and 2 show typical results obtained in Exp. 1 for oxidation of linoleic and linolenic acids by normal rats. In 3 hours the carbohydrate-fed rats converted about one-sixth as much of the injected, labeled, polyunsaturated fatty acids to CO_2 as did the fasted rats. Values for the specific activities (c.p.m. per mg CO_2 carbon) of expired CO_2 were considerably lower in the fed than in the fasted rats at all time intervals.

In Exp. 2, oxidation of linoleic acid was compared in fed normal and diabetic rats. The latter ingest about twice as much food as do normal rats, but in spite of this, the cumulative $C^{14}O_2$ and specific activity values for each time interval in the experiments with diabetic rats were much higher than the corresponding values observed with the normal rats (Fig. 3).

The effect of insulin upon oxidation of linoleic and linolenic acids by diabetic rats was studied in Exp. 3, and typical results are shown in Figs. 4 and 5. Injections of the hormone reduced the conversion of the C^{14} of the 2 labeled, polyunsaturated fatty acids to CO_2 to about the same extent as did carbohydrate feeding in the normal animal.

The results obtained with other rats in each of the 3 experiments are summarized in Table I. Conversion of polyunsaturated to saturated fatty acids has been shown to occur to only a limited extent(19). In view of the short period involved in our experiments, it would therefore seem that we are dealing with the actual oxidation of the administered fatty acids.

Discussion. Despite the fact that the capacity for linoleic acid synthesis is either severely limited or lacking in the rat, the characteristics of the oxidation of this fatty acid resemble those of palmitic acid. Thus, it is more rapidly oxidized in the fasted than in the carbohydrate-fed rat; its oxidation in the fed diabetic rat greatly exceeds that in the fed normal rat; and the increased oxidation in the diabetic rat can be reduced by insulin administrations. The evidence obtained here indicates that oxidation of linoleic and linolenic acids, like that of palmitic, is tied directly to glucose utilization and indirectly to insulin

availability. Our findings on the comparative rates of oxidation of linoleic acid-1-C^{14} by the fed normal and diabetic rats are compatible with the observations of Peifer and Holman (17), that the alloxan-diabetic rat is more rapidly depleted of essential fatty acids than is the normal rat.

Summary. 1. Oxidation of linoleic and linolenic acids, both randomly labeled with C^{14}, to CO_2 was compared in a) fasted and glucose-fed normal rats; b) normal and diabetic fed rats; and c) insulin-treated and untreated, fed diabetic rats. 2. Administration of glucose spared oxidation of both linoleic and linolenic acids. 3. Oxidation of linoleic acid by *ad lib.*-fed diabetic rat exceeded that by similarly fed normal rat. 4. Insulin spared oxidation of polyunsaturated fatty acids in the diabetic rat.

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Conversion of Serum Proteins into Tissue Proteins.* (25120)

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Although elimination of isotopically labeled plasma proteins from the circulation has been investigated by numerous authors(1-9), very little is known on their conversion into tissue proteins. The purpose of the experiments described here was to gain more insight into this process. Whipple and his coworkers(1,10) in their classical work with C^{14}-lysine containing plasma proteins found incorporation of C^{14}-lysine into the tissue proteins. It is not possible, however, in experiments with proteins containing a single labeled amino acid to decide whether incorporation into tissue protein involves transfer of large peptide fragments, or whether total breakdown to the amino acid stage is a prerequisite for incorporation. External labels such as I^{131} are use-

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less in experiments of this type since they are not utilized in formation of tissue proteins. More information can be obtained when the injected plasma proteins contain 2 or more labeled amino acids. Obviously the ratio of the 2 labels remains unchanged when large peptide fragments are transferred from plasma protein to tissue protein. Conversely, a drastic change in the ratio of the 2 labels would result when breakdown to small fragments were followed by incorporation of the fragments at different rates. In our experiments we injected rats with rat serum proteins biosynthetically labeled by C^{14}- and S^{35}-amino acids.

Methods. Preparation of doubly labeled rat serum proteins. A C^{14}-amino acid mixture was obtained by hydrolysing the proteins of a *Chlorella* culture grown‡ on $C^{14}O_2$. After removal of humin by centrifuging the aqueous solution was purified by chromatography on a Dowex-50 column to remove any carbohydrate that might have been present. The preparation of S^{35}-yeast protein and its hydrolysis has been described(11). Approximately 6.4×10^7 counts per minute (CPM) of the C^{14}-amino acid mixture and 1.5×10^8 CPM of the S^{35}-amino acid mixture were combined and injected intraperitoneally into a rat of 106 g weight which had been starved 12 hours prior to injection. The animal was exsanguinated by heart puncture after 5 hours. After dilution of the serum with 3 volumes of 0.9% saline the globulins were salted out by 50% saturation with ammonium sulfate at pH 8.6 in the presence of 0.4% cysteine hydrochloride. They were electrophoretically free of albumins and contained 4800 CPM/mg of C^{14} and 10,500 CPM/mg of S^{35}. The proteins of the supernatant albumin fraction were salted out by saturation with ammonium sulfate, dissolved in and dialyzed against distilled water. They contained 2940 CPM/mg of C^{14} and 9020 CPM/mg of S^{35}. Electrophoresis revealed the presence of 10-20% globulin. Rat serum albumin containing S^{35} and I^{131} was prepared by the method used for preparation of rabbit serum albumin-S^{35},I^{131} (11). *Injection of rats and analysis of organ proteins.* Rats weighing 100 to 180 g were injected into their femoral veins with the radioactive proteins. Rats Nos. 1, 2 and 3 received 9.2, 17.7 and 29.6 mg serum albumin-S^{35},C^{14}; rats Nos. 4 and 5 were injected with 7.5 and 19.7 mg of serum globulin-S^{36},C^{14}. Control rats were injected with rat serum albumin-S^{35},I^{131}. The animals were exsanguinated by cardiac puncture, the organs rinsed with saline solution, homogenized in saline and precipitated with trichloracetic acid. Powders of the serum and tissue proteins were prepared and counted as described earlier (11). Hair was washed with water, acetone and ether. The activity due to each isotope in doubly labeled material was calculated from the decay rate(12). Sulfur content of the serum and tissue proteins was determined‡ by the Denis-Benedict method as modified by Waelsch and Klepetar(13).

Results. Following injection of rat serum albumin-S^{35},C^{14} into rats, the 2 isotopes disappeared from the serum at a similar rate as shown by Fig. 1. In the tissue proteins, relative specific activities of S^{35} and C^{14} increased during the first few days from 0 to approximately 0.5, then decreased slowly. Since $S^{35}{}_T/C^{14}{}_T$, the ratio of the 2 isotopes in the tissue proteins, depends on $S^{35}{}_P/C^{14}{}_P$, their ratio in the injected plasma proteins, we express our results as quotients, Q_i of the isotope ratios (Table I):

$$(1) \qquad Q_i = \frac{S^{35}{}_T/C^{14}{}_T}{S^{35}{}_P/C^{14}{}_P} = \frac{S^{35}{}_T \times C^{14}{}_P}{S^{35}{}_P \times C^{14}{}_T}.$$

The activities $S^{35}{}_T$, $S^{35}{}_P$, $C^{14}{}_T$, and $C^{14}{}_P$ were measured in CPM per mg of protein. According to Table I the values of Q_i vary from 0.9 to 4.6 after injection of the doubly labeled rat serum albumin, and from 1.1 to 6.7 after injection of the doubly labeled globulin. The highest values of Q_i were found in the hair.

Discussion. If the conversion of plasma proteins into tissue proteins involved merely the transfer of large peptide fragments from the injected protein to tissue proteins, the ratio $S^{35}{}_T/C^{14}{}_T$ in the tissue protein would be equal to the ratio $S^{35}{}_P/C^{14}{}_P$ of the injected

‡ We are indebted to Drs. Sam Hood and Gunter Zweig of Kettering Inst., Yellow Springs, O., for growing *Chorella* in C^{14}-carbonate which we received from Oak Ridge National Labs. We are also grateful to Frank Zaboretzky for carrying out the sulfur analyses.

TABLE I. Relative S^{35}/C^{14} Ratios (Q_i)* and Corrected S^{35}/C^{14} Ratios (Q_c)* in Rat Organ Proteins after Injection of Rat Serum Albumin-S^{35}, C^{14} (RtSA) and Rat Serum Globulin-S^{35}, C^{14} (RtSG).

Rat No.		SF-1		SF-3		SF-4		SF-5	
Injected		RtSA (1.4 % S)		RtSA (1.4 % S)		RtSG (0.91 % S)		RtSG (0.91 % S)	
Killed after (days)		3		9		3		9	
Protein	S %	Q_i	Q_c	Q_i	Q_c	Q_i	Q_c	Q_i	Q_c
Serum†	1.1	1.19		1.15		.89		1.00	
Lungs	.97	1.10	1.59	1.26	1.59	1.55	1.44	1.34	1.25
Heart	1.0	.87	1.22	1.07	1.50	1.27	1.14	1.60	1.44
Muscle‡	.97	1.29	1.85	1.17	1.69	1.15	1.07	1.44	1.33
Spleen	.88	1.26	2.02	.96	1.53	1.26	1.29	1.40	1.43
Hair	3.8	3.95	1.44	4.63	1.71	6.77	1.60	5.90	1.40
Liver	.97	1.18	1.70	1.14	1.65	1.97	1.82	2.14	1.98

* Q_i = cpm S^{35}/cpm C^{14} in tissue protein divided by cpm S^{35}/cpm C^{14} in inj. protein; $Q_c = Q_i \times$ % S in inj. protein divided by % S in tissue protein.

† Q_c values for serum cannot be calculated since the serum contains the inj. as well as newly formed protein(11,14).

‡ From the shoulder region.

proteins and Q_i (in equation 1) would be 1.0. The increase of Q_i to values as high as 6.7 demonstrates clearly that the transfer of large peptide fragments cannot be the general metabolic pathway. The high values of S^{35}/C^{14} in the hair protein indicated a relation to the high sulfur content of hair keratin. We determined, therefore, the percentage of total sulfur in the injected plasma proteins (S_P) and in the analyzed tisue proteins (S_T), respectively. Substituting in equation 1 the corrected S^{35}-activities (CPM per mg of protein sulfur) for the measured S^{35}-activities (CPM per mg of protein) we obtain a "corrected quotient of the isotope ratios" Q_c (equation 2). Since the carbon content of all proteins is close to 50-55%, the C^{14}-activities per mg of protein carbon must be proportional to the C^{14}-activities per mg of protein; hence no correction is necessary for the C^{14}-activities.

(2) $Q_c = Q_i \times (S_P/S_T)$

The mean value of Q_c in tissue proteins of rats injected with serum albumin-S^{35}, C^{14} is 1.64 ± 0.20; in animals injected with rat serum globulin Q_c is 1.43 ± 0.25. Hence our results are represented by equation 3:

$$(3) \qquad \frac{S^{35}{}_T}{C^{14}{}_T} = Q_c \frac{S^{35}{}_P}{C^{14}{}_P} \times \frac{S_T}{S_P}.$$

Equation 3 demonstrates first that $S^{35}{}_T/C^{14}{}_T$ in proteins of the tissues is proportional to $S^{35}{}_P/C^{14}{}_P$ in the injected proteins. While this is not surprising, the equation also shows that $S^{35}{}_T/C^{14}{}_T$ is proportional to S_T, the percentage of sulfur in the examined tissue protein, but inversely proportional to S_P, the total content of sulfur in the injected protein. This suggests strongly breakdown of the injected serum proteins to the amino acid stage, dilution of the amino acid pool by both non-radioactive and radioactive amino acids of the injected plasma proteins, and incorporation of free amino acids at a rate proportional to the sulfur and carbon content of the newly formed tissue proteins.

The factor Q_c may vary for different amino acid mixtures and may also depend on the animal species. Its magnitude of approximately 1.5 reveals an impoverishment of C^{14} relative to S^{35} during the conversion of plasma protein into tissue protein. As Penn *et al.*(15) have shown, this may be due to the preferential utilization of essential amino acids in the biosynthesis of new proteins. A considerable portion of the dispensable C^{14}-amino acids is converted into lipids, carbohydrates and other metabolites which are not formed from cystine and methionine.

Our view that utilization of injected plasma proteins involves their breakdown to the amino acid level is further supported by comparing the metabolic fate of serum albumin-S^{35},I^{131} with that of serum albumin-S^{35},C^{14}. Although the elimination of S^{35} from the serum and its incorporation into the tissue proteins is very similar in both proteins (Fig. 1), the metabolic fate of I^{131} which is not utilized for protein synthesis in the analyzed organs is quite different from that of S^{35} and C^{14}. The

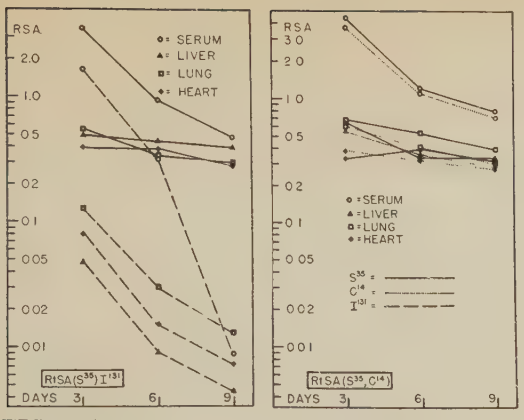

FIG. 1. Relative specific activities (RSA) of protein-bound S^{35}, C^{14} and I^{131} in tissues of rats, 3 to 9 days after intrav. inj. of doubly labeled rat serum albumin. Left: Inj. of rats #6, 7, and 8 with rat serum albumin-S^{35}, I^{131}. Right: Inj. of rats #1, 2, and 3 with rat serum albumin-C^{14}, S^{35}. The relative specific activity is defined as cpm in 100 mg protein divided by cpm inj./g body wt.

quotient Q_i (equation 1), which is equal to $(S^{35}{}_T/I^{131}{}_T)/(S^{35}{}_P/I^{131}{}_P)$ and which in rabbits injected with rabbit serum albumin-S^{35}, I^{131} in 9 days increases to values from 3 to 14 (11), approaches in rats injected with rat serum albumin-S^{35},I^{131} values from 50 to 100 (Figure 1). These high values reflect the higher metabolic rate of rat serum proteins as compared with rabbit serum proteins.

Summary. 1. Incorporation of S^{35} and C^{14} from injected doubly labeled rat serum albumin or globulin into the tissue proteins of rats was investigated. The ratio S^{35}/C^{14} in tissue proteins varies very little during 9 days after injection of the doubly labeled serum proteins. 2. The ratio S^{35}/C^{14} in tisues is approximately equal to $(S^{35}{}_P/C^{14}{}_P) \times (S_T/S_P) \times Q_c$ where the first term is the ratio of the 2 isotopes in the injected plasma protein, S_T and S_P the per cent of total sulfur in the analyzed tissue protein and the injected plasma protein, respectively, and Q_c is approximately 1.5. - 3. The increase in S^{35}/C^{14} ratio ($Q_c > 1$) indicates impoverishment of C^{14} relative to S^{35} and is attributed to utilization of C^{14}-amino acids for other metabolic pathways. 4. In rats injected with serum albumin-S^{35}, I^{131} the ratio S^{35}/I^{131} increases during 9 days from 50 to 100 times. 5. Our results indicate that conversion of plasma proteins into tissue proteins involves breakdown to amino acids or very small peptide fragments. There is no indication for the transfer of large peptide fragments from plasma protein to tissue protein molecules.

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Experimental Phenylketonuria in the Monkey.* (25121)

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Our previous report(1) indicated that plasma phenylalanine and tyrosine levels in rats may be changed by supplying these amino acids to the normal diet. After 2 to 3 months on 2.5% L-tyrosine and/or 2.5% DL-phenylalanine diets, these animals showed marked retardation in temporal discrimination learning. Phenylalanine hydroxylase levels in livers were markedly reduced. It seemed worthwhile to test whether these biochemical effects and learning disability could be produced in monkeys.

Methods and materials. Six adolescent Macacus Rhesus weighing 3 kg each were tested for presence of tuberculosis and dysentery prior to experiment. After short observation, the animals were divided into 3 groups. Two animals received L-phenylalanine, 2 received L-tyrosine, and 2 received L-phenylalanine and L-tyrosine as supplement to their diet every 8 hours. The amino acids were intimately mixed with vitamin preparation,† spread on slice of bread and folded so that no dry material could be spilled when the inquisitive animal unfolded the amino acid-vitamin sandwich. Chim biscuits,‡ fresh fruit and water were also fed daily. Fasting blood samples were drawn from femoral vein into heparinized syringes at biweekly or weekly intervals. Plasma phenylalanine was determined by method of Udenfriend and Cooper (2) and plasma tyrosine analyzed by method of Udenfriend and Cooper (3).

Results. Plasma phenylalanine and tyrosine levels of these monkeys are presented in Table I. An intake of 0.5 g of L-phenylalanine 3 times daily to monkeys IA and IB did not raise the plasma phenylalanine level after 8 weeks. Only when the amino acids offered were increased to 1 g or more, 3 times daily, did the plasma phenylalanine value become significantly elevated. Plasma content gradually decreased after several weeks, but when intake was increased to 2 g or 3 g 3 times daily, markedly elevated plasma phenylalanine levels were found. These high values persisted for a longer period with higher daily intakes. The plasma tyrosine levels in these 2 monkeys paralleled the increased phenylalanine values which was to be expected on the basis of increased conversion of phenylalanine to tyrosine. During this period these 2 animals excreted up to .78 g of phenylpyruvic acid daily in the urine. This is within the range reported in phenylketonurics by other workers(4,5,6,7). This observation is also in accordance with work of Armstrong(8) who found phenylpyruvic acid excretion to occur only if blood phenylalanine value was 15 mg or more/100 ml plasma.

Phenylalanine levels were unchanged in IIA and IIB despite increasing dosages of L-tyrosine; plasma tyrosine levels were variable, with occasional very high plasma level. These animals were sacrificed after 6½ months and their tissues preserved for pathologic study. Urines from these 2 animals failed to show increased phenylpyruvic acid at any time during experiment.

The 2 monkeys IIIA and IIIB receiving both amino acids, did not show as striking elevations in plasma phenylalanine as in monkyes fed L-phenylalanine alone. Only after 3 g each were fed 3 times daily, was the high phenylalanine observed, but this effect was short-lived since near normal values were found 2 weeks later. However, tyrosine values were higher than in IA and IB because tyrosine was added in the diet and because phenylalanine was converted to tyrosine. Phenylpyruvic acid was excreted in urine when plasma levels were elevated.

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† 4 cups powdered milk, 22 g Parvo (Lederle), 1 lb. lard, Cecon (Abbott), 50 ml (1 ml = 100 mg ascorbic acid), Irrodal A. (Parke-Davis) 2¾ lb.

‡ Chim Biscuits (Kennel Food Supply, Hygrade Food Products Mfg. Co., Fairfield, Conn.).

TABLE I. Plasma Phenylalanine and Tyrosine in Monkeys Receiving Amino Acids.

	Diet + L-phenylalanine IA		Diet + L-phenylalanine IB		Diet + L-tyrosine IIA		Diet + L-tyrosine IIB		Diet + L-phenylalanine + L-tyrosine IIIA		Diet + L-phenylalanine + L-tyrosine IIIB	
Weeks	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine
	mg/100 ml											
0	1.7	1.0	1.7	1.0	1.7	1.0	1.7	1.0	1.7	1.0	1.7	1.0
	0.5 g L-phenylalanine T.I.D.*				0.5 g L-tyrosine T.I.D.				0.5 g each amino acid T.I.D.			
4	1.5	1.8	1.3		1.4		1.6	1.6	1.8	2.8	1.8	3.1
8	1.9	2.7	1.8	1.8	1.2	1.5	1.0	1.2	.8	2.5	1.4	3.0
9	1.0 g L-phenylalanine T.I.D.				1.0 g L-tyrosine T.I.D.				1.0 g each amino acid T.I.D.			
11	15.0	11.0	9.1	7.5	.6	5.8	1.0	13.4	3.8	15.3	5.6	20.6
15	4.9	5.8	5.8	4.3	1.4	3.0	1.5	5.0	2.3	7.0	1.8	7.4
17	1.5 g L-phenylalanine T.I.D.				1.5 g L-tyrosine T.I.D.				1.5 g each amino acid T.I.D.			
20	11.7	7.3	1.8	2.7	1.5	3.2	1.4	5.5	1.6	6.0	1.8	12.6
25	3.0 g L phenylalanine T.I.D.				3.0 g L-tyrosine T.I.D.				3.0 g each amino acid T.I.D.			
27	24.7	6.4	19.2	7.2			1.2	>60	6.4	>60	5.5	
29	36.0	10.2	20.2	5.2					26.6	50	20.6	31.6
31	24.8	12.5	4.6	6.9					2.7	1.6	2.5	3.9
33	15.5		13.7									

* Three times daily.

The extraordinary high plasma phenylalanine values may be simply the result of overloading metabolic capacity of the phenylalanine hydroxylase enzyme system. In addition, the possibility exists that amount of activity of this enzyme was actually decreased by phenylalanine or by products derived from it, as has been demonstrated in rats in this laboratory (unpublished results).

Plasma tyrosine levels were highest in those monkeys which received both tyrosine and phenylalanine. In these animals both dietary tyrosine and conversion of phenylalanine to tyrosine contributed to the elevated level. When the phenylalanine decreased, it was reflected in lower tyrosine values.

The present data indicate that it is possible to produce in monkeys elevated plasma phenylalanine levels as well as urinary excretion of phenylpyruvic acid similar to that in clinical condition of phenylpyruvic oligophrenia. Psychological experiments now in progress will test intellectual performance of such animas as well as their motor development.

Summary. 1. Prolonged and sustained elevated phenylalanine plasma values have been observed in monkeys fed 3 g of L-phenylalanine 3 times daily. 2. Phenylpyruvic acid was excreted in the urine of these animals. 3. The biochemical findings are comparable to those found in phenylketonuria.

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Relationship Between Mevalonic Acid Utilization and ATP Content in Liver Homogenates Pretreated with Ribonuclease.* (25122)

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Preincubation of whole rat liver homogenates with crystalline ribonuclease abolishes the capacity of these homogenates to convert mevalonic acid (MVA) into non-saponifiable material or cholesterol(1). Homogenates that have been inactivated by ribonuclease may be restored to full synthetic capacity by autoclaved extract of fresh liver and existence in such heated liver extracts of an unknown factor essential directly or indirectly for MVA utilization was postulated(2). It was suggested that the factor may be concerned with phosphorylation of MVA since in homogenates pretreated with ribonuclease added MVA remains in a microbiologically determinable form(1,3). Evidence is here presented that liver homogenates pretreated with ribonuclease are devoid of ATP while homogenates suitably fortified with liver extract contain ATP. The factor contained in liver extract appears to be concerned with a functional level of ATP which, in turn, is required for phosphorylation of MVA(4,5,6).

Methods and materials. The experimental procedures involved in biosynthetic studies have been described(7). In brief, liver from young rats is homogenized with a loose homogenizer and centrifuged briefly to remove connective tissue. Aliquots of the essentially whole homogenate fortified with DPN but not with ATP are preincubated with and without ribonuclease and autoclaved liver extract alone or in various combinations. Perchloric acid extracts are prepared after preincubation for ATP determinations. Paired flasks are then supplemented with labelled MVA and reincubated for a determination of capacity of treated homogenates to synthesize non-saponifiable material from the labelled precursor. Flasks are aerated with oxygen prior to preincubation and again at the time of MVA addition. Perchloric acid extracts were prepared by treating the homogenates with an equal volume of 4% perchloric acid. Excess perchloric acid was removed as the insoluble potassium salt. ATP was determined spectrophotometrically following adsorption and gradient elution of the extracts on Dowex-1-formate. Details involved in the preparation of extracts and chromatographic separation were as described by Allfrey and Mirsky(8). Following incubation of flasks containing added MVA-2-C^{14} the contents were saponified, extracted with petroleum ether, the extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted (7). Autoclaved liver extract was prepared by treating fresh guinea pig liver in the cold with twice its weight of water in a Waring Blendor for about a minute. The brei was then autoclaved at 105-110° for 20 minutes and the mixture cooled and centrifuged at 10,000 x g for 20 minutes. The coagulum was discarded. Dowex-1-treated liver extract was prepared by passing a liver extract solution over a large excess of Dowex-1 in the chloride form.

Results. Optical densities at 260 mμ of the fractions obtained by ion exchange chromatography of perchloric acid extracts of a control and a ribonuclease-treated homogenate of liver are presented in Fig. 1. The curves are typical of quite a group and show that at the time of MVA addition to preincubated homogenates ribonuclease-treated preparations in contrast to controls contain no ATP and do not incorporate MVA into non-saponifiable material. In Table I are presented similar data from a number of experiments. Included are the results from a few experiments where inactivation of MVA utilization as evidenced by counts found in the non-saponifiable fraction was not complete (See Exps. 1, 2 and 5). Such incomplete inactivation is sometimes encountered and is probably attributable to biological variability in the homogenates. Where counts in the ribonuclease-treated flasks were

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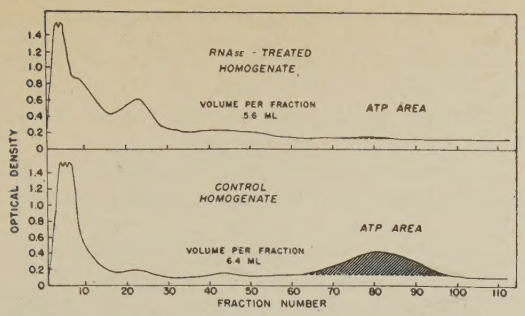

FIG. 1. ATP content of rat liver homogenates incubated with and without ribonuclease. Activity of the non-saponifiable material isolated from a duplicate control flask to which MVA-2-C^{14} was added at the time the first flask was treated with perchloric acid in preparation for chromatography was 5,450 cpm. Activity of the non-saponifiable material isolated from a duplicate ribonuclease-treated flask similarly treated was 17 cpm.

only slightly reduced ATP was present in amounts comparable to that found in control homogenates.

The results of a typical experiment illustrating the ATP encountered when liver homogenate was preincubated with and without ribonuclease and autoclaved, Dowex-1-treated liver extract in 4 possible combinations are presented in Table II. Given also are the CPM found in paired flasks to which MVA-2-C^{14} was added following preincubation which were allowed to continue incubation for a measure of MVA utilization. It is apparent from the data that homogenates preincubated with ribonuclease and treated liver extract are active in biosynthesis of labelled non-saponifiable material and that they also contain significant amounts of ATP. This is in contrast to homogenates preincubated with ribonuclease alone where MVA utilization is negligible and no ATP is detectable. As sometimes happens control homogenates without ribonuclease are more active with respect to MVA utilization and ATP content when supplemented with liver extract (compare flasks 1 and 2). When the data of Table II are examined graphically where the counts found in the non-saponifiable fraction are plotted against ATP concentration at the time of labelled MVA addition without regard for the treatment the homogenates received a straight line with positive slope and zero intercept results. Thus in the system under study the level of ATP at time of MVA addition determines extent of MVA utilization.

TABLE I. Effect of Preincubation with Ribonuclease on ATP Content of Homogenates and Subsequent Biosynthesis of Labelled Non-Saponifiable Material (NSF) from MVA-2-C^{14}.

Exp.	Ribonuclease (mg)	Preincubation (min.)	ATP (mg)	NSF (cpm)
1	0	30	1.86	4,010
	5	"	1.38	2,980
2	0	90	.82	3,000
	10	"	.11	1,640
3	0	40	1.66	5,450
	10	"	.08	17
4	0	45	2.27	9,237
	8	"	.04	17
5	0	"	2.98	7,765
	10	"	.32	1,411

TABLE II. Effect of Preincubation with Ribonuclease and Autoclaved Liver Extract in Various Combinations on ATP Content of Homogenates and the Subsequent Biosynthesis of Labelled Non-Saponifiable Material (NSF) from MVA-2-C^{14}.

Ribonuclease (mg)	Supplement	ATP (mg)	NSF (cpm)
0	None	1.30	6,175
0	5 ml autoclaved liver extr.	2.02	9,190
10	None	.0	28
10	5 ml autoclaved liver extr.	.56	2,990

Discussion. Presumably in the aerobic homogenate system employed in these studies a steady state level of ATP exits that is a balance between the ATP originating largely from oxidative phosphorylation and the ATP undergoing phosphate transfer to various substrates or hydrolysis by ATPases. Preincubation with ribonuclease in the presence of "tissue fragments," previouly shown to be essential for the ribonuclease effect, appears to inactivate the cycle by hydrolyzing an essential polynucleotide. Thus rat liver homogenates preincubated with ribonuclease contain essentially no ATP while similar homogenates supplemented with an autoclaved extract of liver from which ATP has been removed by anion exchange chromatography prevent or reverse ribonuclease inactivation by supplying this essential polynucleotide factor. Since according to current concepts phosphorylation of MVA by mevalonic kinase in the presence of ATP is the first step in biochemical conversion of this compound to non-saponifiable material

the failure of ribonuclease-treated homogenates to utilize MVA becomes interpretable. Data presently available do not permit a decision as to the locus of action of the essential polynucleotide. Involvement of polynucleotides in ATP biosynthesis has been previously observed in thymus cell nuclei pretreated with *deoxyribonuclease* by Allfrey and Mirsky(8) and in fractionated extracts of *Alcaligenes fecalis* by Pinchot(9). A number of recent reviews have been concerned with the existence of postulated intermediates in reactions involving biosynthesis, conservation, or utilization of ATP(10,11,12).

Summary. Ribonuclease-treated homogenates of liver that do not incorporate mevalonic acid into non-saponifiable material or cholesterol were devoid of ATP. ATP was present and mevalonic acid incorporation occurred when ribonuclease pretreatment was carried out in presence of an autoclaved extract of liver. The results are interpreted as indicating existence of a polynucleotide factor essential for maintenance of a positive balance with respect to ATP in liver homogenates.

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